STUDIES ON THE MORPHOLOGY, INNERVATION, AND GROWTH OF SKELETAL MUSCLE FIBRES

A Thesis submitted for the
degree of Doctor of Philosophy
of the University of Edinburgh
by Bruce Mackay, M.B., Ch.B. (Edin.)

1960

CONTENTS

The main object of this study has been a comparative survey of the distribution of motor neuromuscular junctions in vertebrate skeletal muscle, and the principal method employed has been the histochemical demonstration of these as sites of cholinesterase concentration.

In so vast a field as the skeletal musculature of vertebrates, some discrimination has been necessary in the selection of material for study. The observations are therefore presented in three sections.

In the first section, a study is made of the distribution of motor endplates in the segmental musculature of a representative series of lower vertebrates, from Amphioxus to reptiles. Observations of cholinesterase distribution have been supplemented by silver staining and electron microscopy to give a fuller picture of the morphology and innervation of segmental muscle.

The second section deals with avian muscle. As will be discussed, the muscles of birds appear to be built up of two varieties of muscle fibre which differ in their mode of innervation. The attempt has been made, therefore, to demonstrate these two forms of innervation by histochemical means.

The patterns of endplate distribution in a series of mammalian muscles are described in the third section, together with certain additional observations made to try to

determine the significance of some forms of endplate distribution.

In the discussion which follows, the histochemical demonstration of cholinesterase in skeletal muscle is first considered, and then the results of the three sections are, in turn, compared with those of previous workers, and their significance assessed. The effectiveness of the combination of techniques used in the present work is evaluated, and the occurrence of cholinesterase at muscle-tendon junctions is examined. From the literature, instances are quoted of the main reasons that have been put forward in favour of the subdivision of skeletal muscle fibres into two distinct types, and it is considered how far the results of the present studies may fit in with these hypotheses.

Utilising certain information gained in the course of this work, a series of experiments was carried out to try to determine the site, or sites, on a skeletal muscle fibre at which growth in length takes place. These experiments are described, and the results considered, in the final section of the Thesis.

Methods

Two histochemical procedures were employed for the demonstration of sites of cholinesterase concentration in skeletal muscle. The first was a modification of the technique first described by Koelle and Friedenwald (1949), using acetylthiocholine as substrate. Results obtained with this method vary considerably with alterations in a number of factors which influence the staining reactions, notably the process of fixation, pH of the incubating medium, and length of incubation; to obtain satisfactory results and avoid staining artefacts, a preliminary series of tests is necessary for the muscle of each individual species in order to determine the optimum conditions for the staining process. Where material was limited, as in the case of certain of the lower vertebrates studied, a series of sections was stained simultaneously at different pH values for varying lengths of time, and the best sections selected.

The basic procedure followed was to fix material in 10% formalin solution, if possible by perfusion but otherwise by immersion of small blocks of tissue in the solution, for a period of about one hour. Material could then be stained as whole mounts, or in small teased pieces, or as frozen sections. The incubating medium in this technique has

poor penetrating power, and apart from thin sections, only the surface of a preparation is stained. It was found that perfusion of material with the incubating solution, where this was possible, would permit staining of cholinesterase sites at all depths through the preparation, but a considerable quantity of incubating solution is required, and this procedure was rarely employed.

Following fixation, material was washed in distilled water to remove the formalin, and was then immersed completely in the incubating medium, which had been buffered to a suitable pH. Most satisfactory results were obtained using a sodium acetate - acetic acid buffer in the pH range pH 4 to pH 6.5; above pH 6.5, a tris-(hydroxymethyl)-amino-methane buffer was employed. The composition of the incubating solution was :

Acetylthiocholine iodide	15 mgm.
Copper sulphate 1% solution	0.3 ml.
Distilled water	0.75 ml.

The supernatant fluid from this solution after the precipitate had settled, was added to a second solution whose composition was :

Buffer solution	5 ml.
Distilled water	3.8 ml.
Copper sulphate 1% solution	0.2 ml.
Glycine 3.7% solution	0.2 ml.

Incubation was always carried out at room temperature. After a suitable period of incubation, determined by removing small test pieces, the preparation was removed, washed briefly in distilled water, and immersed in 5% ammonium sulphide solution in order to convert the white precipitate at sites of enzyme activity to black copper sulphide; a few seconds was usually all that was required for this step. Material was then further washed, this time in running tap water, and whole mounts were stored in glycerol. Small pieces of tissue, and frozen sections, were dehydrated and mounted on slides in Canada balsam.

The second histochemical procedure employed was the modification by Lewis (1958) of the standard azo-dye procedure. This was found in many instances to give more satisfactory results than the acetylthiocholine technique, but since the azo-dye is soluble in higher alcohols, it was necessary to mount preparations in glycerol. As Lewis (1958) observes, considerable distortion can be produced in small pieces of tissue by transferring directly from an aqueous medium into glycerol, and small strips of tissue or frozen sections were therefore passed through a series of glycerol-alcohol-water mixtures before finally being mounted in pure glycerol.

Apart from the use of eserine described in the section on avian muscle, selective inhibitors were not employed to characterise the cholinesterase stained by the above methods.

For silver staining, material was fixed in acetic-formol-alcohol (glacial acetic acid 5 ml.; formol 5 ml.; alcohol 90 ml.). Paraffin sections were cut at 10 μ or 15 μ, and stained with a silver nitrate - egg albumen method (Peters, 1958).

Electron microscopy was carried out on small pieces of tissue fixed for one hour at 4° C. in 1% osmic acid adjusted to pH 7.2 with either chromate-dichromate buffer (Dalton, 1955) or veronal-acetate buffer (Palade, 1952). After fixation, specimens were washed in chilled 10% ethanol, dehydrated rapidly in graded ethanols, and embedded in methacrylate. Thin sections were cut with a Porter-Blum "Servall" microtome, using a glass knife, and were examined with a Metropolitan-Vickers E.M. 6 electron microscope.

Additional methods, where used, are described in the text.

Observations

1. Segmental Muscle of Lower Vertebrates.

The simplest organisation of vertebrate skeletal muscle fibres is into segmental myotomes, and it is probable that the somatic musculature of early vertebrates consisted entirely of a series of myotomes running from head to tail. In present day animals, however, such an arrangement is only found in Amphioxus. Here, the myotomes are V-shaped, and as in all segmental muscle, the muscle fibres are aligned parallel to the long axis of the body, and attached at their ends to the myosepta, which form fibrous partitions between the adjacent blocks of muscle. In the cyclostomes, this simple segmental pattern has been lost at the cephalic end of the animal, and in the remainder of the body the myotomes have assumed a W-shape. With the appearance of paired fins in the higher fishes, the original myotomal arrangement is further disturbed, but it is nevertheless retained in an almost unchanged form in the greater part of the body in both fish and Urodeles. In the Anura, which are predominantly terrestrial, myotomes are only present in the tail of the tadpole, and it is in the tail that they persist in the reptiles. The loss of the simple myotomal structure, first in the head and later in the limb regions, thus becomes almost complete on the assumption of a terrestrial existence, and simple myotomes are only present in the embryonic forms of

birds and mammals.

The types of innervation found in the myotomes of lower vertebrates merit study since they may represent the prototypes from which the nerve-muscle relationships of higher vertebrates have evolved. In this work, the myotomal muscle from at least one example of each of the major groups of lower vertebrates has been studied, primarily by observing the distribution of sites of cholinesterase concentration. Further observations on the morphology and innervation of these muscles was made using silver staining and electron microscopy, this being necessary in a number of instances to explain the significance of the cholinesterase distribution.

Observations were made on the following species :

Cephalochordata :	Amphioxus	(Amphioxus lanceolatus).
Cyclostomata :	Lamprey	(Lampetra planeri) (Lampetra fluviatilis)
Elasmobranchii :	Dogfish	(Scyliorhinus canicula)
Teleostei :	Goldfish	(Carassius auratus)
	Loach	(Nemachilus barbatulus)
	Salmon	(Salmo salar)
	Trout	(Salmo trutta)
	Minnow	(Phoxinus laevis)
	Stickleback	(Gasterosteus aculeatus)

Urodela :	Salamander	(Salamandra salamandra)
	Newt	(Triturus viridescens)
	Pleurodele	(Triturus waltlii)
Anura :	Frog	(Rana temporaria)
	Xenopus	(Xenopus laevis)
Reptilia :	Grass snake	(Tropidonotus natrix)
	Green lizard	(Lacerta viridis)

Cephalochordata

Amphioxus

In frozen sections of Amphioxus muscle stained to demonstrate sites of cholinesterase activity, the staining material shows up as a series of diffuse streaks running through the myotomes parallel to the muscle fibres (figs. 1 - 3). In most cases these streaks do not extend to the myosepta, but on occasion they can be seen to do so (fig. 2). Otherwise, staining is not seen in a myoseptum, apart from where a nerve bundle is present (fig. 1). Scrutiny of sections stained with each of the cholinesterase techniques has failed to show any further sites of enzyme localisation within the myotomes.

To seek the explanation for this cholinesterase distribution, a study of silver-stained sections was undertaken. In longitudinal sections it is possible to follow the muscle fibres through the whole extent of a myotome. The fibres are slender, and appear to be loosely bound together (fig.4). Under higher magnification, however, some variation in the calibre of individual fibres can be seen.

Serial transverse sections show that the dorsal and ventral nerve roots emerge alternately from the spinal cord, and there is no connection between them. The dorsal roots run direct through myoseptal connective tissue to the skin, and branches to the myotomes have not been observed. Ventral roots emerge from the spinal cord as wide thin plates,

and course over the medial aspects of the myotomes, giving small branches which appear to enter the myotomes directly, as well as larger divisions which pass into the myosepta. Within the myosepta, these nerves fan out, and bundles of fibres enter the myotomes to supply the muscle fibres. Nerves destined for the lower ventral part of a myotome can be followed as a bundle through its upper part (fig. 4 - arrow).

It has been found difficult to follow the finer nerves within the myotomes. Silver staining has produced little contrast between these fine nerves and the muscle fibres, (Ayers, 1921, speaks of the "translucent, silver-grey colour" of the nerves), and on account of the narrow calibre of each, the distinguishing criterion must be the striation of the muscle fibres which itself may be difficult to discern. At high magnification, however, small dark spots may be seen scattered amongst the muscle fibres, often triangular in outline, and frequently with a fine thread running to them (figs. 5 and 6 - arrows). These probably represent the terminal expansions of the motor nerves.

Electron microscopy of Amphioxus muscle shows that the bundles of myofilaments (corresponding to what were described as muscle 'fibres' with the light microscope) may vary greatly in calibre (figs. 7 and 8). Areas can be seen in which all the bundles present show a similar girth, but in other sections there are wide differences, and a narrow bundle

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may expand to become a relatively wide sheet. Older workers (Grenacher, 1867; Schneider, 1902; Ayers, 1921) describe these bundles as muscle 'plates', and this concept is supported by the recent electron microscopical studies of Bone (personal communication) and Peachey (1960); Peachey describes them as "lamellar muscle fibres less than 1 μ thick, more than 100 μ wide, and stretching the length of the myotome". The irregular appearance seen in electron microscopic sections could be the result of buckling and curving in the course of preparation of the tissue, and these distorting effects are likely to be considerable with methacrylate embedding.

Fig. 9 shows the muscle plates as they are attached to the fibrous tissue of a myoseptum. Clefts similar to those found in higher vertebrates are present, but on account of the narrow diamater of many of the plates there is often room for only a single cleft (fig. 9 - arrow). Where a wide aspect of a plate meets a myoseptum, several clefts are present.

The question arises - are these muscle plates themselves the muscle fibres, or are they component myofibrils of larger muscle units? Sarcolemmal sheaths have not been observed round these bundles of myofilaments, and no connective tissue partitions are seen subdividing a myotome. Relatively few nuclei are present, and there is little connective tissue to be seen; mitochondria are placed between the myofilament bundles (fig. 10) and have not been observed within them.

It seems possible, therefore, that these bundles of myofilaments are in fact myofibrils, and components of a muscle 'fibre' which may extend over the whole myotome. In this connection it is interesting to note that Heymans and Van der Stricht (1898) were convinced that all the muscle plates of a single somite contracted at one time.

A nerve bundle is present in the myoseptum in fig. 9. Neuromuscular junctions have not been observed with the electron microscope.

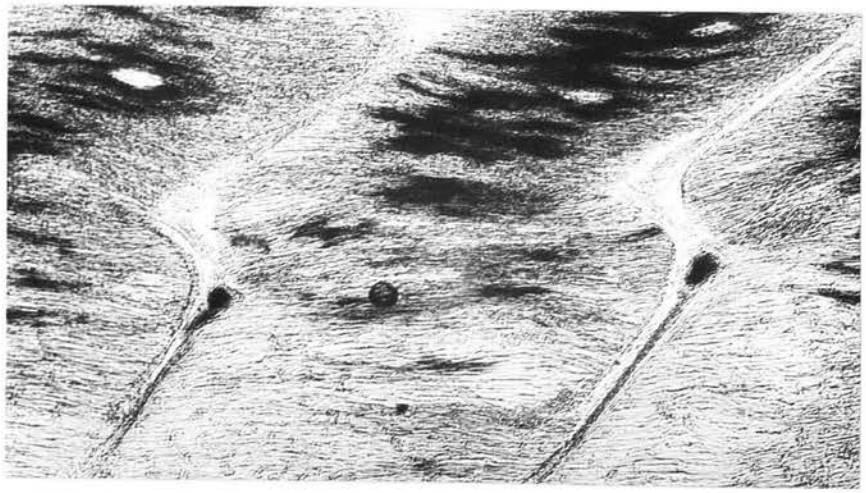


Fig. 1

(x 45)

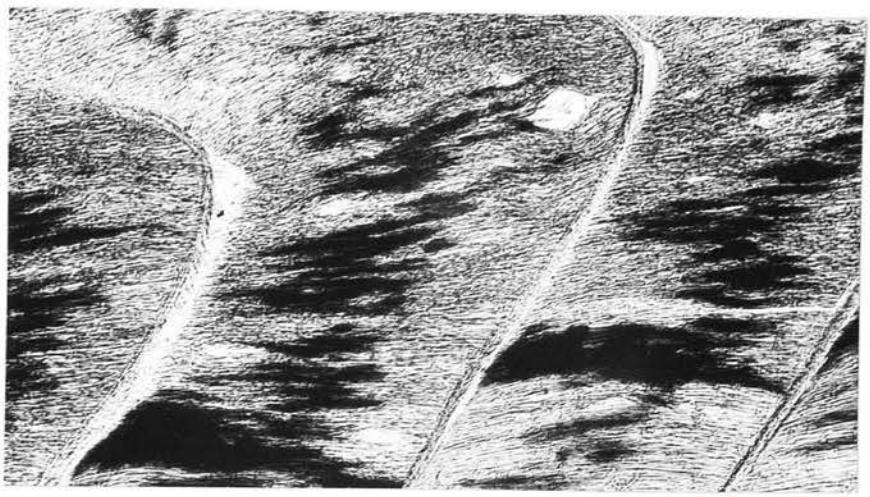


Fig. 2

(x 45)

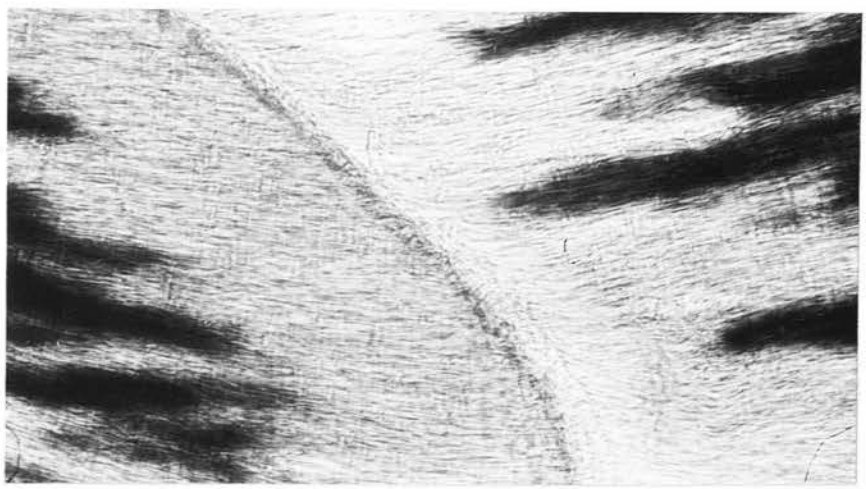


Fig. 3

(x 100)

Amphioxus (azo-dye) : cholinesterase in myotomes.

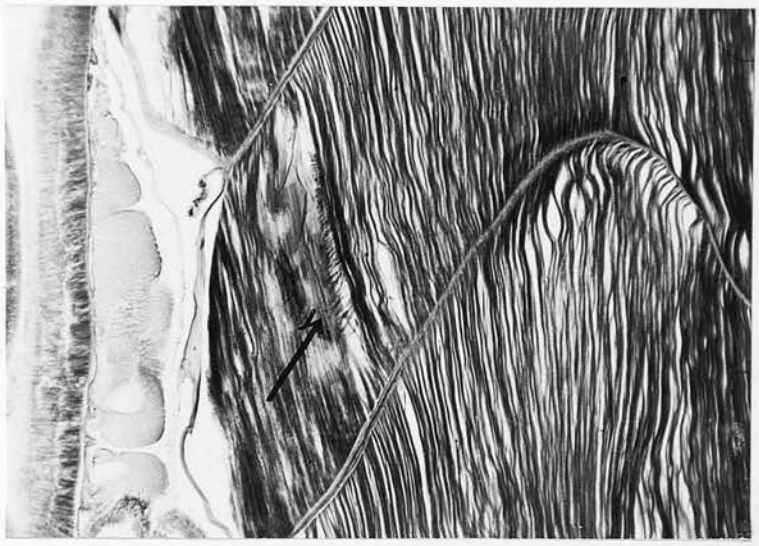


Fig. 4 : Amphioxus (silver) : a bundle of nerves (arrow) can be seen within the myotome. (x 50)

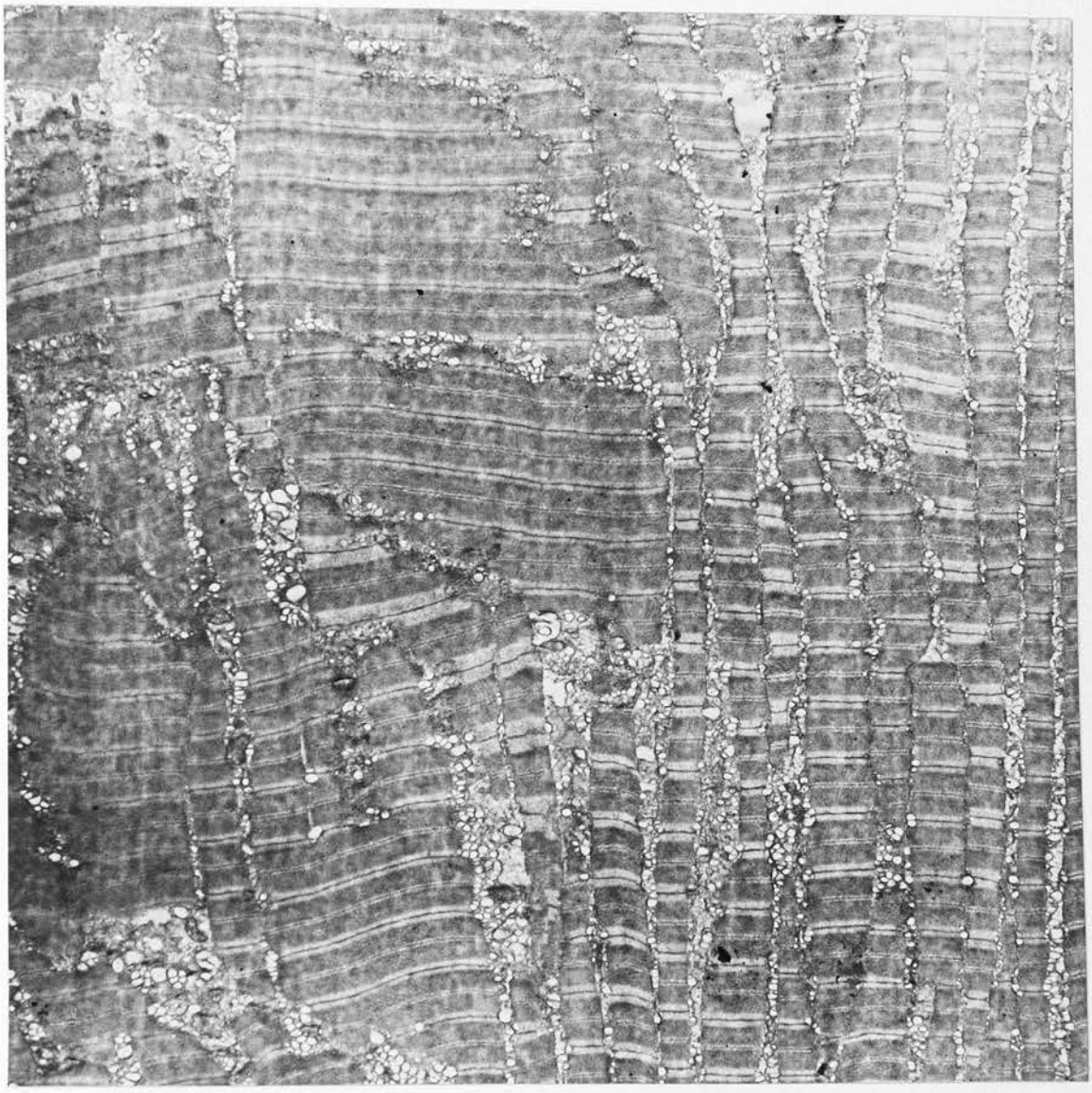


Fig. 7 : Amphioxus (electron micrograph) : myotomal muscle showing variation in calibre of muscle plates. (x 5,000)

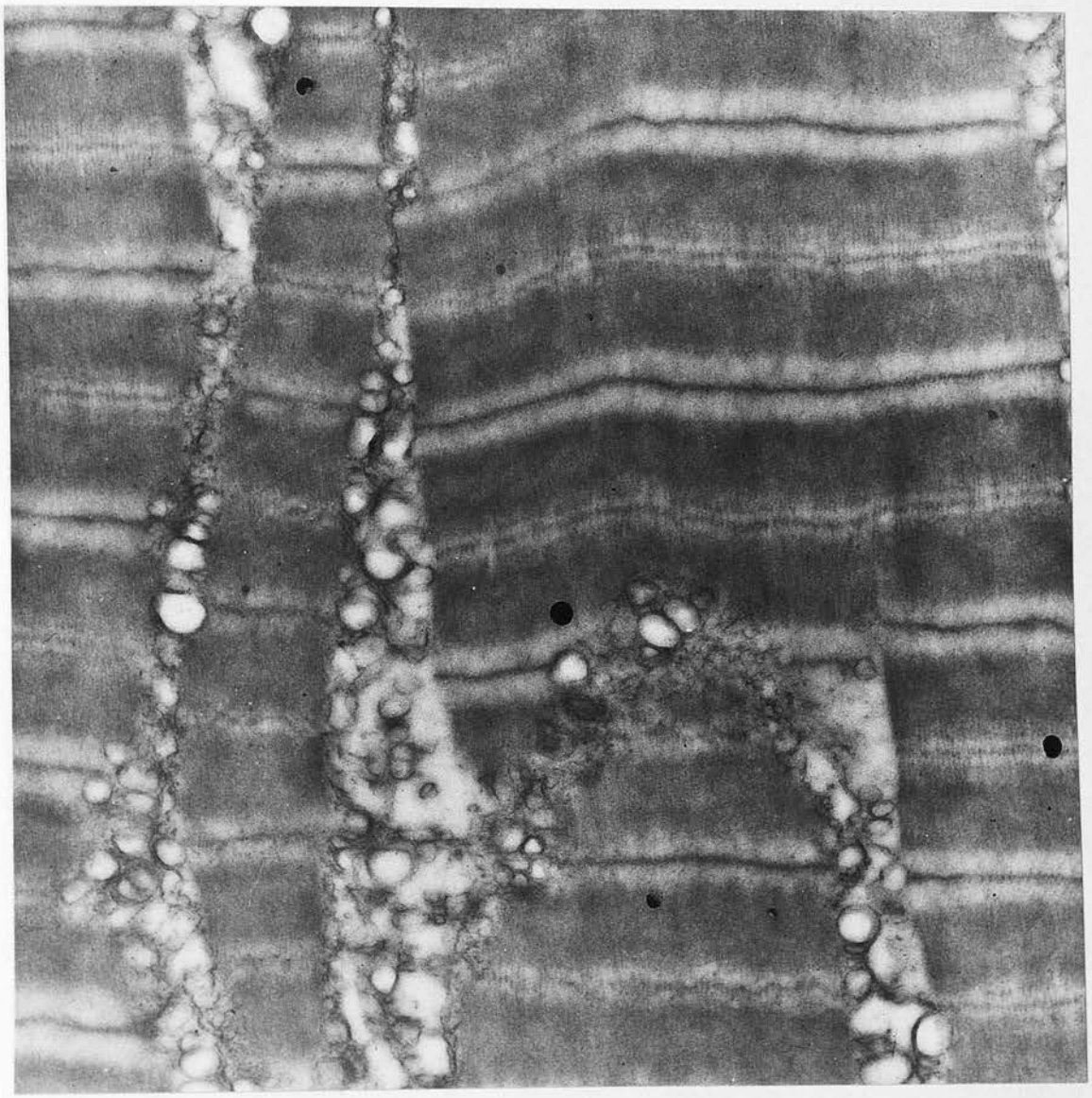


Fig. 8 : Amphioxus (electron micrograph) : similar field to that in fig. 7, but at higher magnification. (x 26,000)

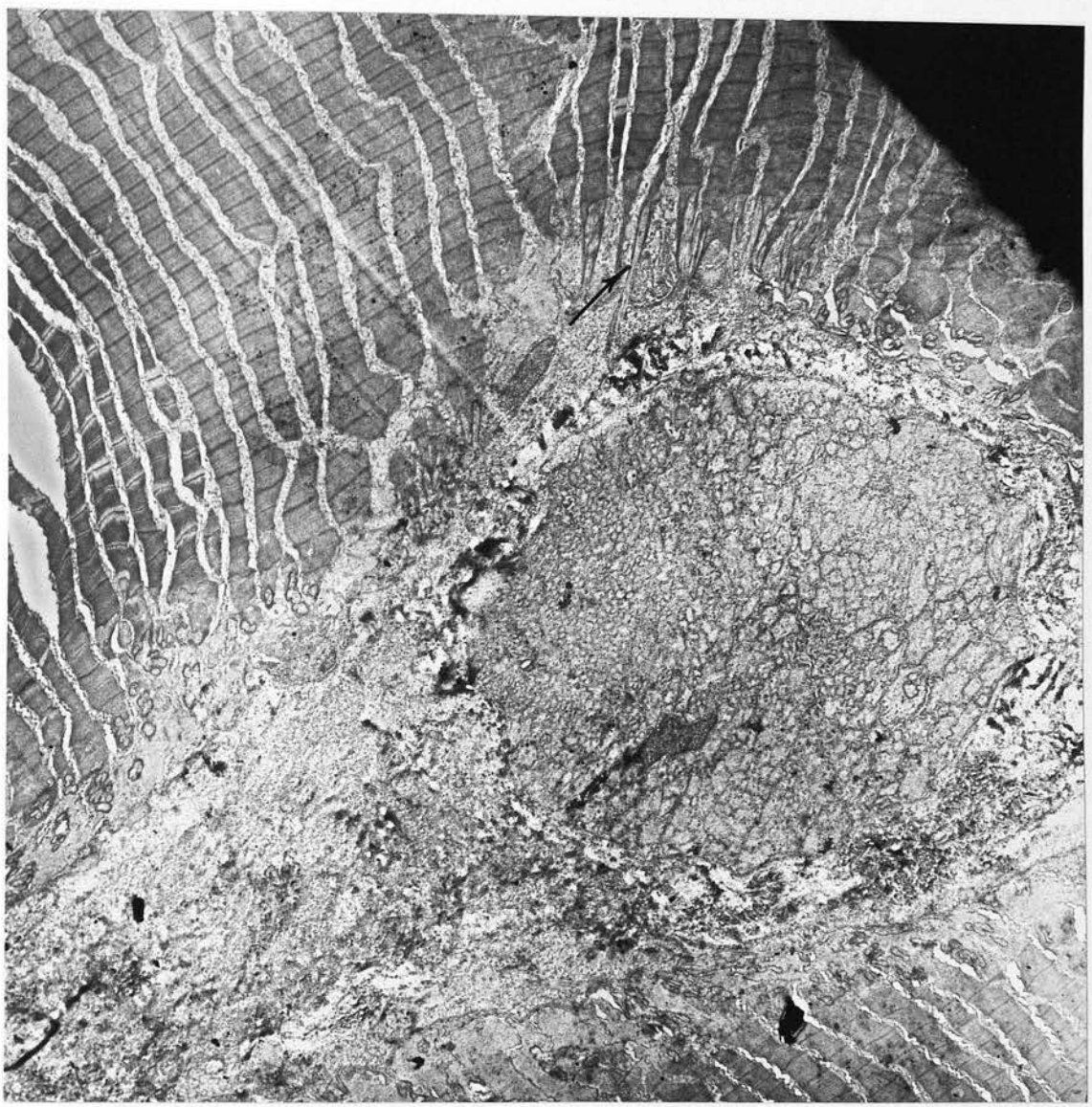


Fig. 9 : Amphioxus (electron micrograph) : a nerve bundle lies within the myoseptum. Clefts at the muscle-tendon junctions can be seen (arrow). (x 3,000).

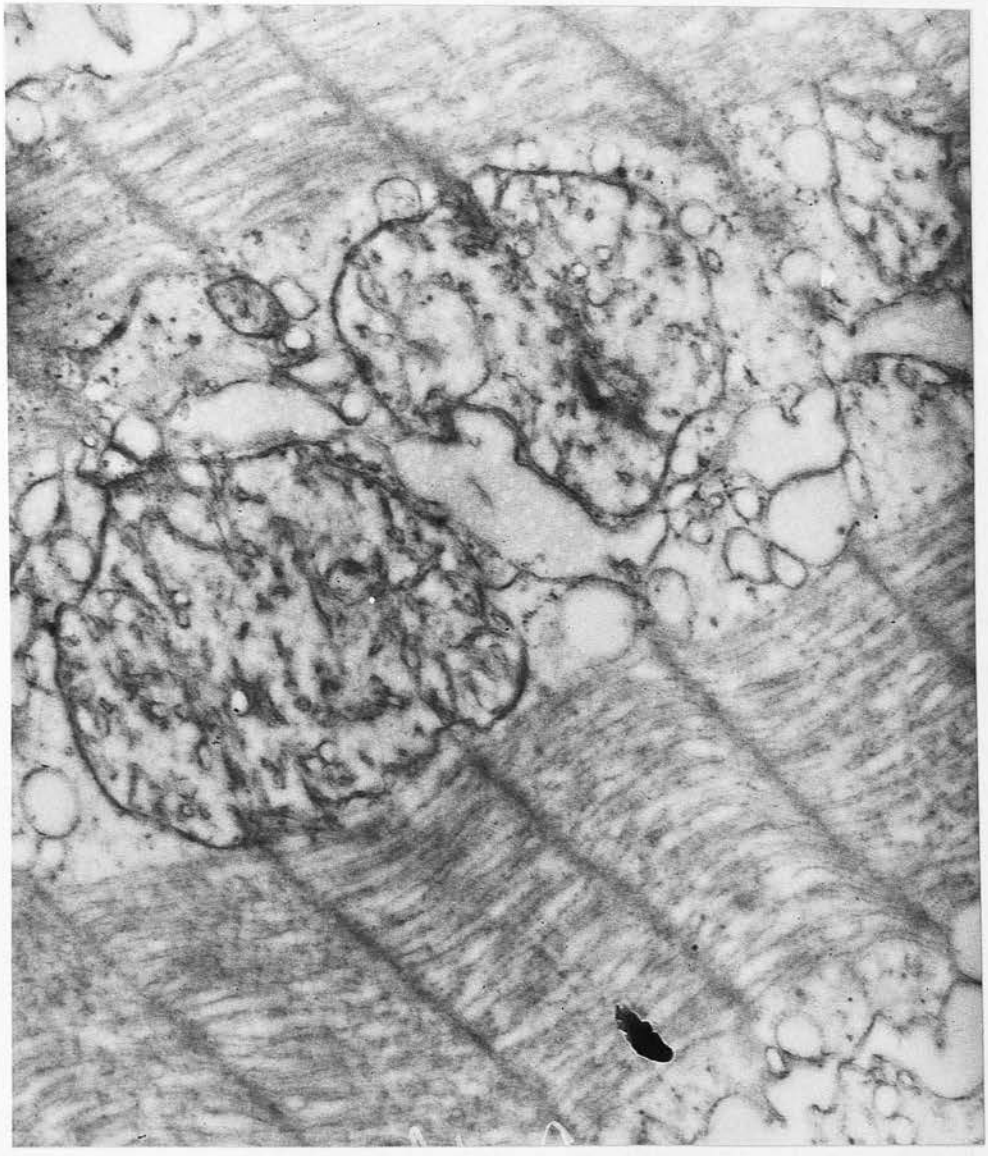


Fig. 10 : Amphioxus (electron micrograph) :
showing two mitochondria. (x 32,000).

Cyclostomata

Lamprey

Frozen sections of the segmental musculature of the lamprey stained for cholinesterase show a bizarre distribution of the enzyme, which can be explained only by relating it to the architecture of the myotomal muscle. The muscle architecture will therefore be discussed first.

Silver-stained sections show that a myotome is composed of horizontally arranged units, each unit extending throughout the myotome from its lateral to medial, and its cephalic to caudal extents. A myotome is built up of a series of such units one above the other. A unit consists of usually six horizontal layers, and the muscle of the dorsal and ventral layers differs in its morphology and innervation from that of the central layers.

The dorsal and ventral layers of a unit are formed by a series of longitudinally arrentated muscle fibres lying side by side; they are somewhat flattened dorso-ventrally, and vary in width. Where the myotome abuts on the skin, these two layers are continuous with one another over the lateral edge of the central layers. This is the only situation in which the side of a unit is bounded by the dorsal and ventral layers coming together; elsewhere the central layers are exposed at the sides of the unit. The muscle of each of the central layers is in the form of an individual sheet of muscle

surrounded by its own sarcolemma (fig. 24). Usually four layers are present, but occasionally, as in fig. 12, only three can be seen. In horizontal sections stained by Wilder's reticulin method, no connective tissue partitions can be seen subdividing a sheet, and although its myoseptal margins may be somewhat crenated, as would be the case if a series of tapering muscle fibres were lying side by side, each sheet is continuous and therefore forms a single muscle plate.

The appearance of a section through the myotomal muscle thus depends on the plane of section. As a myotome extends laterally, it also inclines posteriorly, and consequently more than one myotome is seen in transverse sections (fig. 11). Each myotome shows a stack of cross-sectioned muscle units, and since there is no interval between adjacent units, the appearance is of a regular alternation of four (occasionally three) central plates with two layers of parietal fibres (fig. 12). The myofibrils of the latter appear rather loosely packed in comparison with the texture of the central plates (fig. 13), and while Wilder-stained sections show clearly the connective tissue bounding the parietal fibres and separating them from one another (fig. 14), no connective tissue partitions are visible in the substance of a central plate, though the sheet of muscle is usually broken as a consequence of shrinkage during preparation.

Vertical (parasagittal) sections show that each myotome has the profile of a letter W laid on its side. As in

transverse sections, a regular alternation of four central plates with two layers of parietal fibres is seen, but the parietal fibres are now cut longitudinally and therefore only one is seen in each layer (fig. 15). Since the parietal fibres are shorter than the central plates, the former do not extend into the myosepta, but a sheet of connective tissue connects the layer of fibres above a unit with the layer below, thereby forming a sheath over the anterior and posterior aspects of each quartet of central plates.

Horizontal sections show the posterior inclination of the myotomes as they extend laterally. The arrangement of fibres seen in such sections depends on the level of section, which may pass through central muscle plates, or through a layer of parietal fibres, or if oblique, through both. Where the section passes through a single central sheet over the whole extent of the myotome (fig. 16), it can be seen that the sheet is continuous; no connective tissue partitions can be seen subdividing the layer of muscle into component fibres. A section (fig. 17) through a layer of parietal fibres, or an oblique section showing both varieties of muscle in alternating bands (fig. 18), illustrates the rich innervation of the parietal layers; this is in marked contrast to the paucity of nerves in relation to the central plates, where in fact they are seen only at the myoseptal margins.

The pairs of dorsal and ventral roots in the lamprey emerge alternately from the spinal cord. They do not unite. The dorsal roots run through myosepta to the skin, and branches to the myotomes have not been seen. Each ventral root divides into dorsal and ventral rami which course over the medial aspects of the myotomes. It does not appear that any one root exclusively supplies or runs in relation to any particular myotome, but rather that the main trunk of a nerve continues in a straight course, supplying the myotomes over which that course carries it; each ventral root is therefore distributed to several myotomes. As a ramus runs over the inner face of the myotomes, it gives branches which pass into the myosepta, as well as smaller branches which enter the myotomes direct. These latter nerves run between the muscle units, forming plexuses in relation to the parietal muscle fibres. The central plates are innervated only at their myoseptal margins by short branches from the myoseptal nerves.

The distribution of cholinesterase may now be considered. Fig. 19 shows a frozen section cut in parasagittal plane, and the muscle units are outlined by the zones of stain. Considerable diffusion has occurred, but it can be seen that the enzyme is present at the myosepta and along the parietal fibres, while the bulk of the central plates is free from stain. Figs. 20 and 21 show the region of a myoseptum at higher magnification, and from a similar field in fig. 22, where diffusion has been minimised with shorter incubation,

nerves related to the parietal fibres can be distinguished. Cholinesterase is confined to the myoseptal margins of the central plates, and fig. 23 shows the edge of one plate; small areas where the stain appears to be concentrated can be made out at the periphery of the stained zone (fig. 23 - arrows).

In fig. 24, the ends of two adjacent central plates from a small (4 cm.) Ammocoete are shown, photographed with the electron microscope. A series of myofibrils can be seen extending to the sarcolemma covering the ends of the plates. Mitochondria are numerous towards the ends of these plates, forming densely packed chains between the myofibrils. Elsewhere in the central plates, however, mitochondria are less plentiful, and sarcoplasm is also less abundant. Small clefts can be seen at the ends of the muscle plates, but these are more marked in the adult lamprey (fig. 25). The sarcolemma forms these clefts, and receives attachment of the myofibrils to its inner surface.

As would be expected from the distribution of nerves and of cholinesterase, neuromuscular junctions are only found at the myoseptal edges of the central muscle plates, and they are usually positioned just short of the actual edge, so that they lie close to the line of union between muscle and tendon, where small zones of cholinesterase concentration were observed in fig. 23. In the electron micrograph in fig. 26, a

nerve ending on a central plate is illustrated (E). Part of a second ending is present (E_1). The ending E is covered by a sheath of Schwann cell cytoplasm (S) which is reflected (arrows) to leave the ending bare and in close contact with the muscle sarcolemma. Vesicles present throughout most of the ending are concentrated in this zone. Muscle mitochondria (M) are congregated in the area of sarcoplasm deep to the ending.

Nerve endings may be found at any point along the parietal fibres, and fig. 27 shows an electron micrograph of two adjacent parietal fibres with, lying between them, two nerve endings, one supplying each muscle fibre. The two endings are united by a small bridge (arrowed). Their structure is similar to that of the central plate ending described above. Numerous mitochondria are present within them, and these are smaller and denser than the large, pleomorphic muscle mitochondria. A nucleus (N) lies in the muscle sarcoplasm subjacent to the smaller ending.

Fig. 27 also shows the structure of the parietal muscle fibres. These are distinguishable from the central plates by their more abundant sarcoplasm, by their larger mitochondria, and by a higher content of the colloid-like inclusions (C), whose significance is unknown.

Fig. 11 : Ammocoete (silver) - transverse section showing a ventral nerve root emerging from the spinal cord. Parts of two myotomes can be seen, each a stack of muscle units. (x 50)

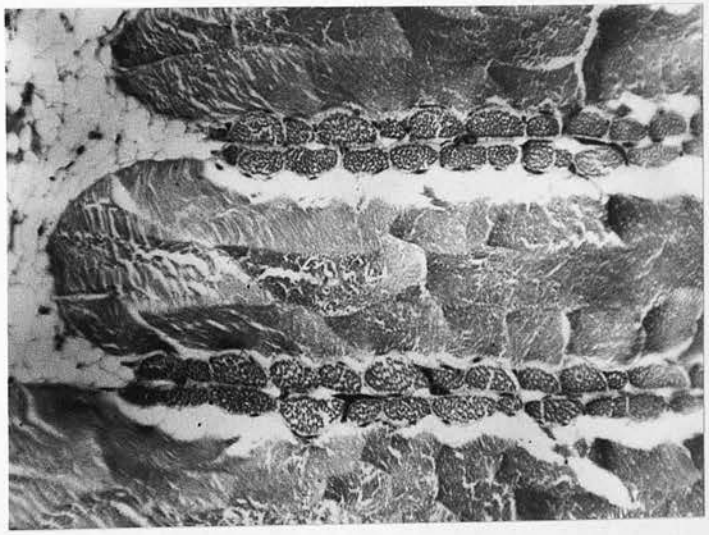


Fig. 12 : Lamprey (silver) - transverse section showing the construction of a muscle unit; only three central plates can be seen between the two double layers of parietal fibres. (x 280)

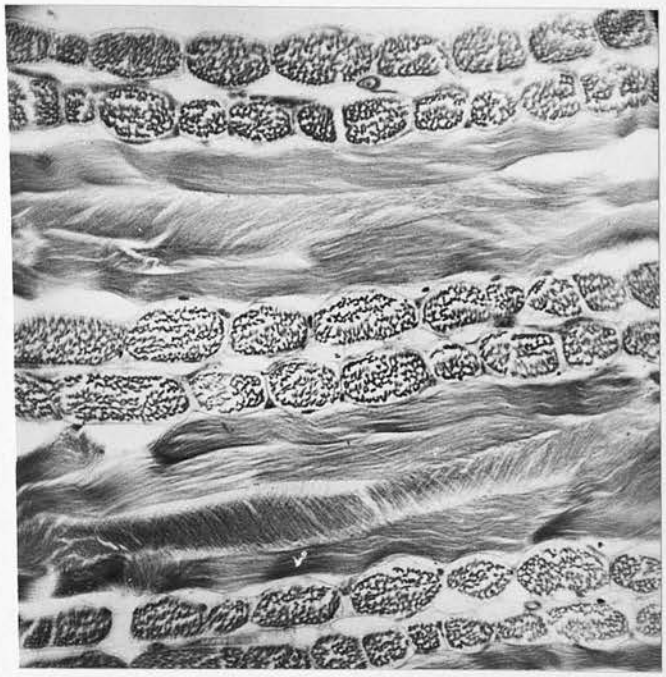


Fig. 13 : Lamprey (silver) - transverse section in which parts of two adjacent muscle units are present; the contrasting textures of the central plates and parietal fibres is evident. (x 330)

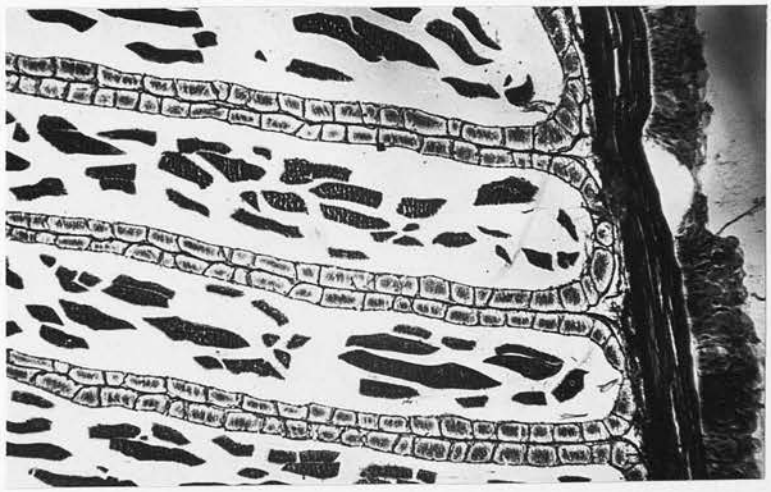


Fig. 14 : Lamprey (Wilder) - transverse section; connective tissue septa subdivide the parietal layers into component fibres; the dorsal and ventral layers of a unit come together deep to the skin. (x 240)

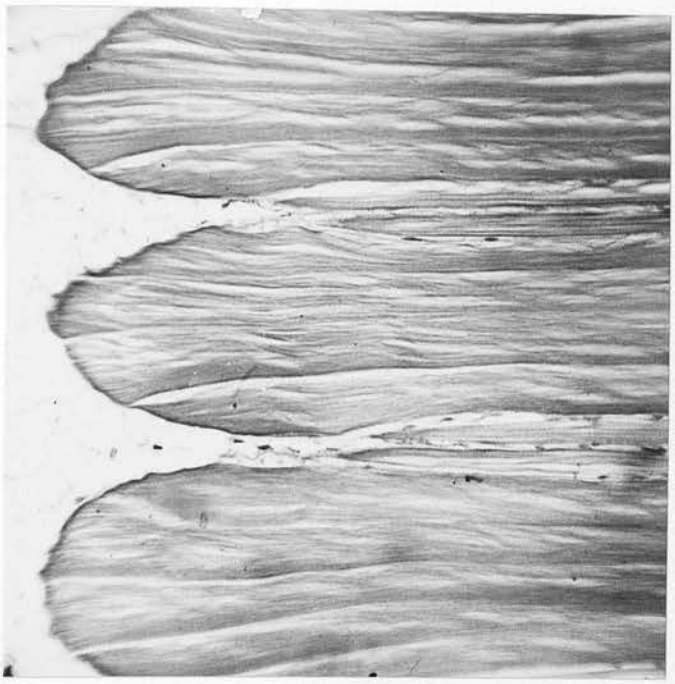


Fig. 15 : Lamprey (silver) - parasagittal section; the parietal fibres are cut longitudinally; they do not bulge into the myoseptum as do the central plates. (x 300)

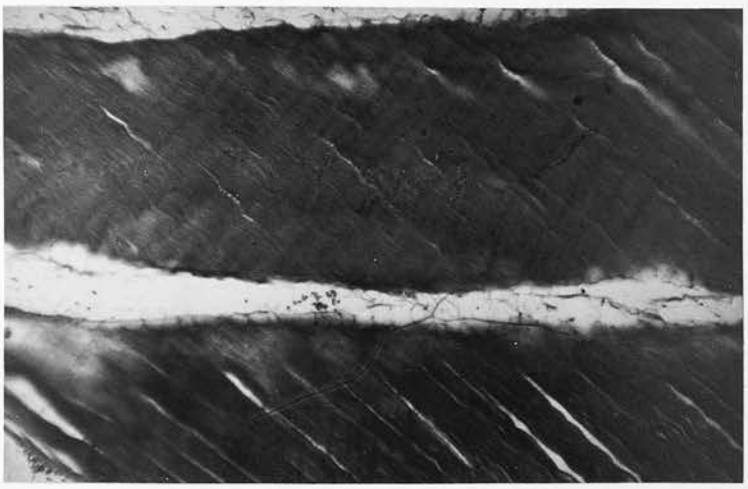


Fig. 16 : Lamprey (silver) - horizontal section passing through individual central plates. (x 210)

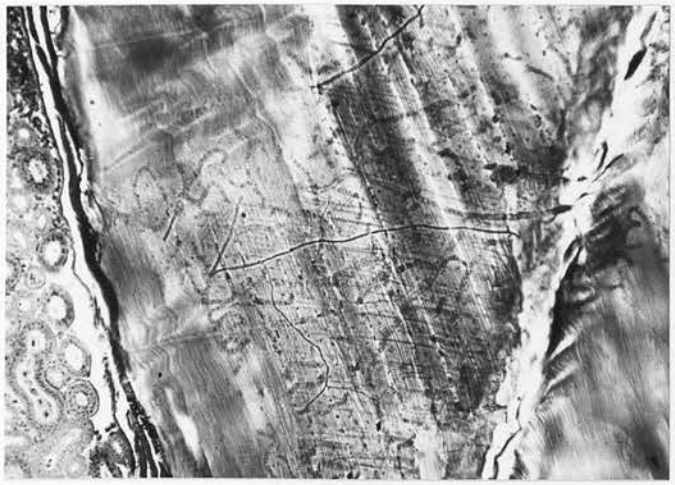


Fig. 17 : Lamprey (silver) - horizontal section mainly through layer of parietal fibres; part of the nerve plexus related to this layer can be seen. (x 490)

Fig. 18 : Lamprey (silver) - oblique horizontal section showing alternating pairs of parietal fibres separated by central plates. (x 510)

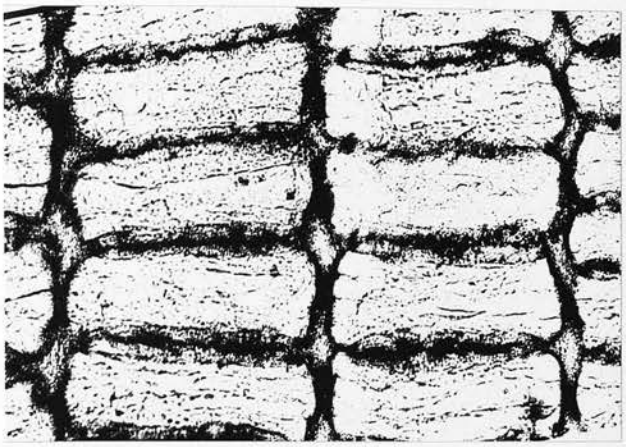


Fig. 19 : Lamprey (acetylthiocholine) - low power view showing distribution of cholinesterase in myotomes; the section is cut in parasagittal plane. (x 54)

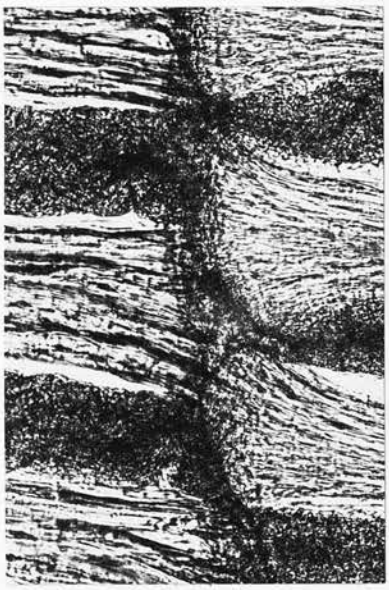


Fig. 20 : Lamprey (azo-dye) - myoseptal region. (x 110)

Fig. 21 : Lamprey (acetylthiocholine) - myoseptal region. (x 110)

Fig. 22 : Lamprey (acetyl-thiocholine) - myoseptal region after short incubation. (x 200)

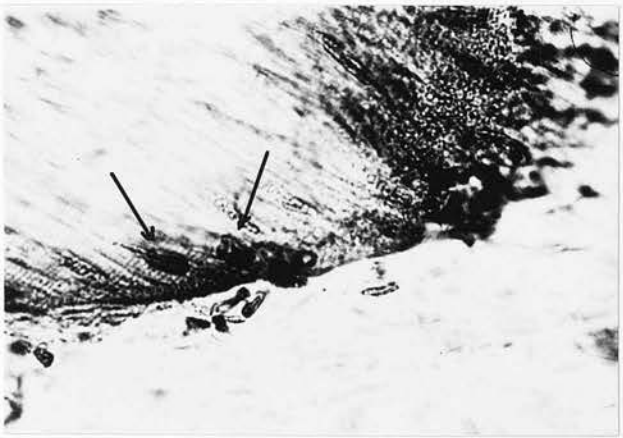


Fig. 23 : Lamprey (acetyl-thiocholine) - showing cholinesterase at the myoseptal margin of a central plate; areas can be seen where the stain appears to be concentrated (arrows). (x 740)

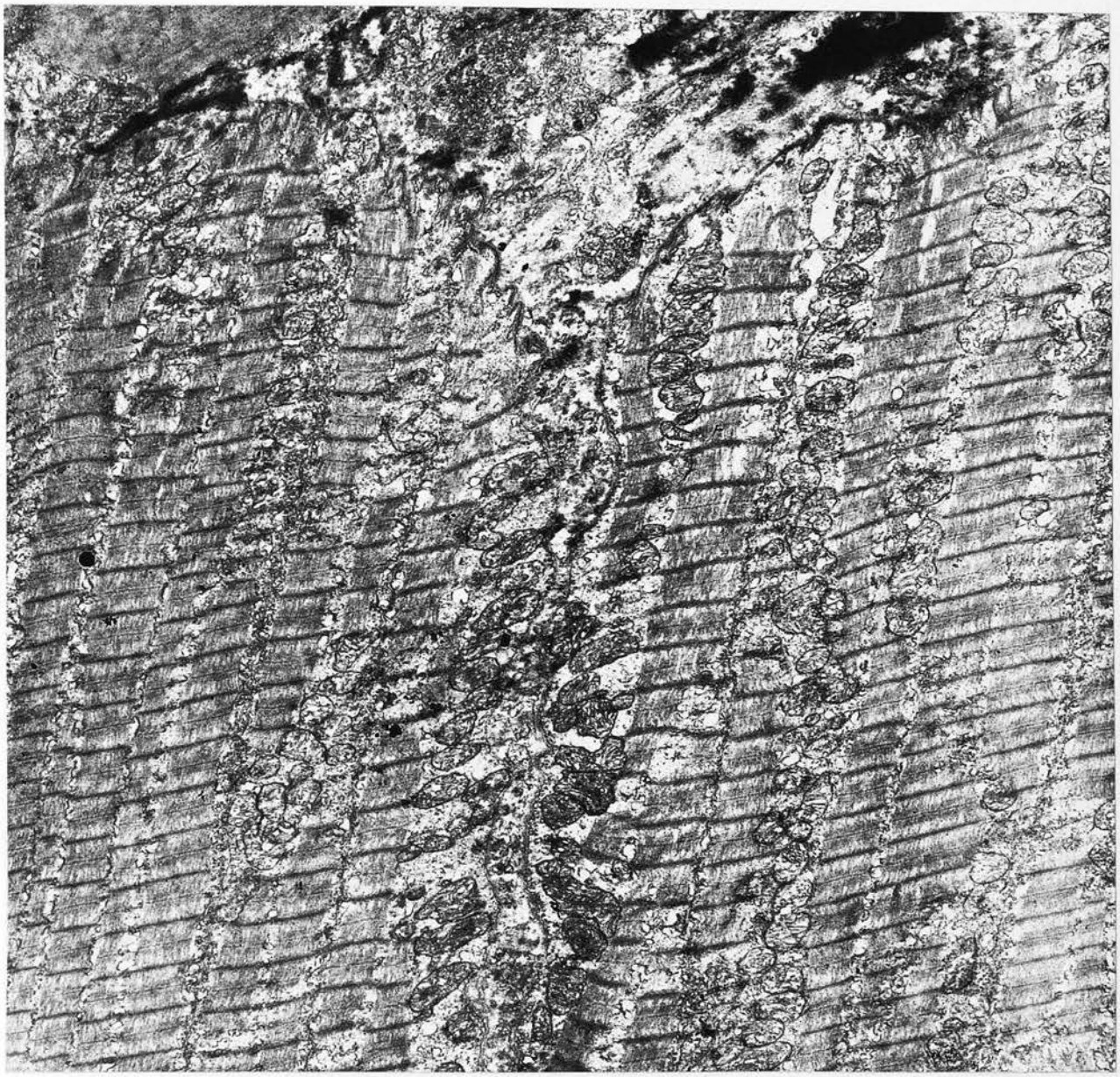


Fig. 24 : Ammocoete (electron micrograph) - showing two adjacent central plates meeting a myoseptum. (x 8,000)

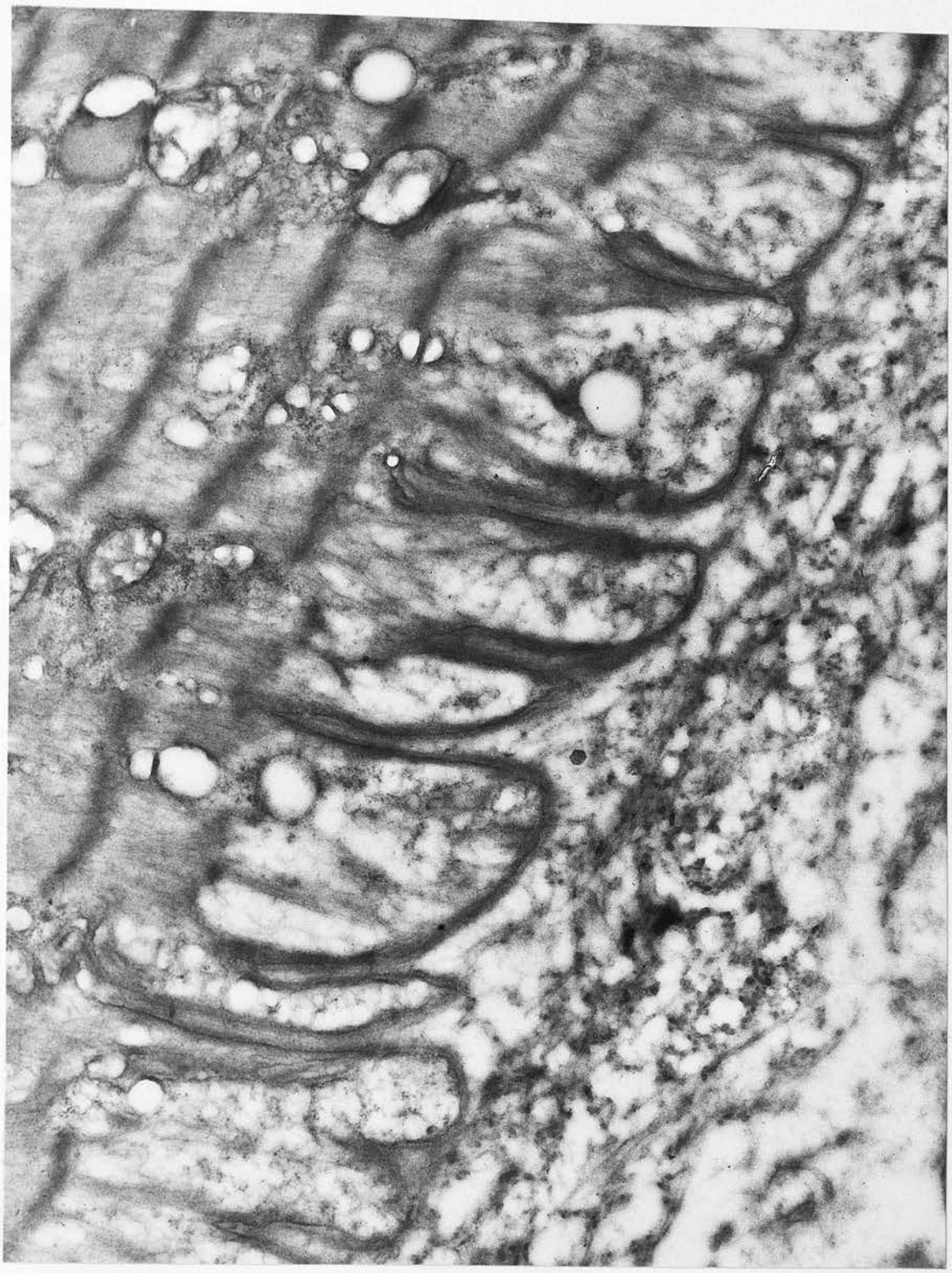


Fig. 25 : Lamprey (electron micrograph) - muscle-tendon junction. (x 20,000)

Fig. 26 : Lamprey (electron micrograph) - nerve ending on a central muscle plate. E = ending; S = Schwann cell sheath, reflected at arrows; M = muscle mitochondria. A second ending can be seen (E_1). (x 31,000)

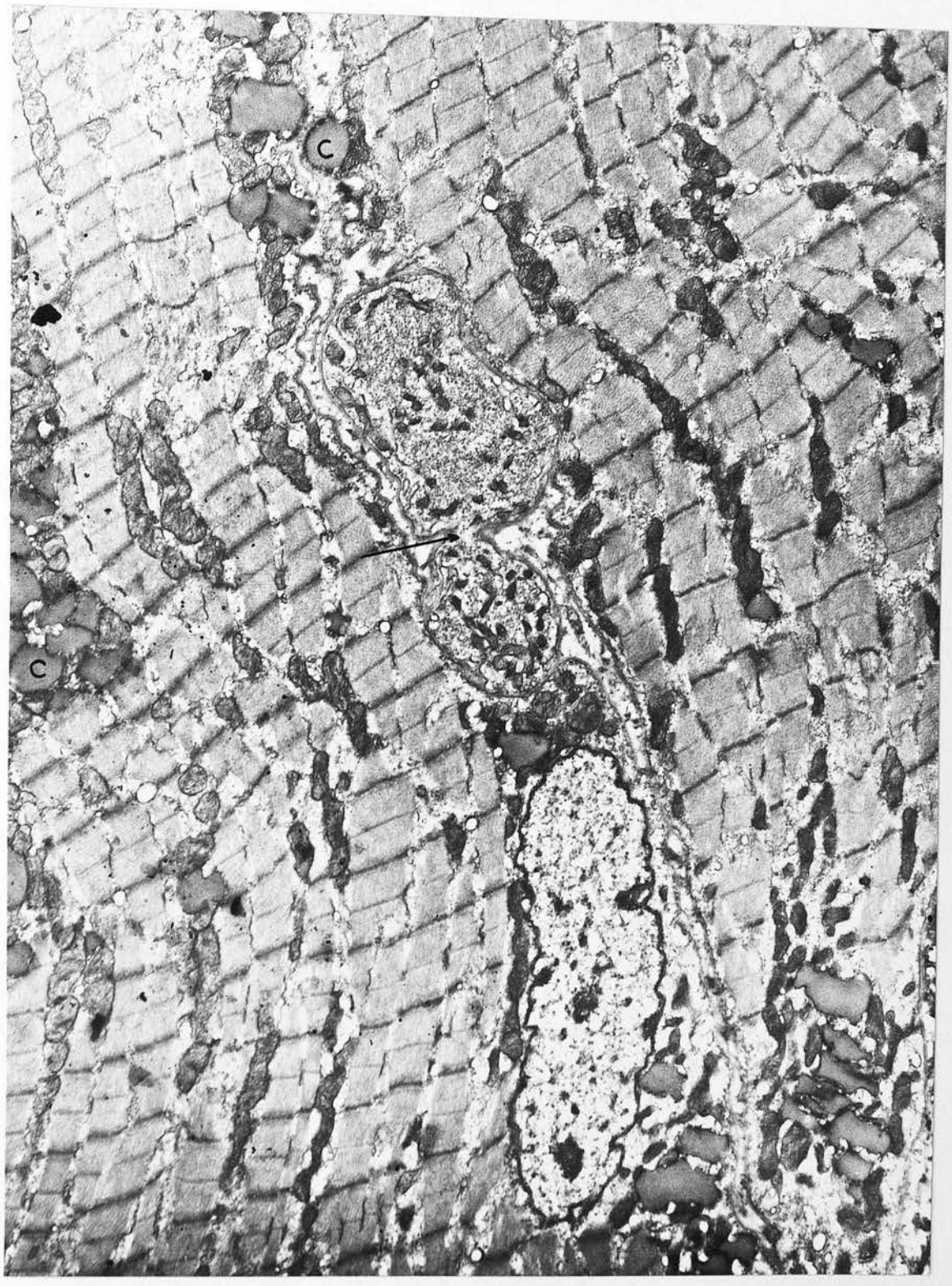


Fig. 27 : Lamprey (electron micrograph) - showing two nerve endings on adjacent parietal muscle fibres; the endings are connected by a bridge of axoplasm (arrow). C = colloid material. (x 20,000)

Pisces

Elasmobranchii

Dogfish

Frozen sections of myotomal muscle from the dogfish proved difficult to stain for cholinesterase. The reason for this is not known, but the patchy distribution of stained areas suggests inadequate fixation. With the azo-dye method, usually effective for cold-blooded animals, only blood cells took up the stain. The acetylthiocholine technique was tried at pH's 4, 5, 6, 7, and staining took place at pH 6, and less satisfactorily at pH 7. Sections incubated at pH 4 or pH 5 did not stain. Fixation was, of necessity, by immersion of blocks of tissue, and frozen sections were cut after the block had been in fixative for 30 minutes. These sections produced staining of neuromuscular junctions at their periphery, and of muscle tendon junctions throughout their whole depth. Sections which were fixed further in formalin solution after being cut, stained poorly or not at all.

Examination of adequately stained sections showed that most of the myotomal muscle is composed of relatively thick muscle fibres. A small zone of more slender fibres was present at the edge of some sections, and these fibres are from the lateral line muscle. In this description, the muscle fibres referred to are the former, unless otherwise stated.

The cholinesterase of the myotomal muscle fibres is only found in the myoseptal regions. The broad fibres are blunt ended, and along the line of contact between a fibre end and the fibrous tissue of the myoseptum, a dark line of cholinesterase is present (fig. 28 - arrow). Many fibres show, in addition, small spots of cholinesterase at their edges near to the end of the fibre; several such spots may be present on one fibre, and adjacent spots may be situated close to one another (lines - figs. 28 and 29). These para-terminal spots are always seen at the surface of the muscle fibre.

Apart from these two sites - terminal and para-terminal - cholinesterase is not found on the myotomal muscle fibre; the major extent of the shaft of a fibre is quite free from stain.

It can be seen from fig. 30 that the cholinesterase in the superficial slender fibres is scattered along the lengths of the fibres, although a prominent myotendinous reaction was also observed. Silver-stained sections (figs. 31) show the transition between the two calibres of muscle fibre, and this transition is fairly abrupt, though the two types are not always separated by a fibrous partition. Nerves can be seen related to the shafts of the slender fibres (fig. 32), but nerves are only found at the ends of the main myotomal muscle fibres.

These observations indicate that the main myotomal muscle fibres in the dogfish are innervated at or near their ends. To determine the site of innervation more precisely, thin

sections from this muscle were examined with the electron microscope. Fig, 33 shows the muscle-tendon junction in a dogfish, with the blunt end of the fibre thrown into a fairly orderly series of sarcolemmal folds. In none of the muscle-tendon junction regions examined has a neuromuscular junction been observed. The structure close to the tip of the muscle in fig. 33 is a capillary, with nucleus (N) and lumen (L).

Fig. 34 shows the sides of the shafts of two adjacent muscle fibres close to, but not actually at, their ends. In the upper part of the field, a nerve ending (E) can be seen indenting the side of one of the muscle fibres. This ending is shown at higher magnification in fig. 35, where it can be seen that the greater part of the surface of the ending in contact with muscle is bare of Schwann cell sheath. Many vesicles are present in the ending.

In the lower half of fig. 34, three nerves can be seen (N, N_1, N_2); it is probable that N_1 and N are different parts of the same axon. Nerve N is seen in longitudinal section, passing down to come in contact with the other muscle fibre, and this zone of contact is shown at higher magnification in fig. 36. A number of vesicles are present in the nerve, but these become more prolific near its termination, and are principally congregated in the zone of the ending which is in direct contact with the sarcolemma. The points of reflection of the Schwann cell sheath are indicated by arrows. A small number of invaginations of varying depth and tortuosity are

present in the muscle sarcoplasm deep to the zone of contact with nerve and ending (figs. 35 and 36 - G).

Teleostei

Cholinesterase distribution was studied in the myotomes of six Teleosts - goldfish, loach, salmon, trout, minnow, stickleback. Silver-stained sections were prepared from goldfish and stickleback, and material from these two species was examined with the electron microscope.

The azo-dye technique gave good results with goldfish, loach, trout, and salmon, but produced no staining in frozen sections of minnow and stickleback. In contrast, the acetylthiocholine method at pH 6 stained sections of minnow and stickleback, but gave less satisfactory results with the other four species.

Cholinesterase distribution in the myotomes of these six fish is illustrated in figs. 37 - 43. Myotendinous staining is never pronounced and is often absent. Most of the enzyme appears as scattered and irregular linear aggregations which may be found at all depths within the myotomes. This is the only pattern of distribution that has been seen in all six species.

Silver-stained sections of goldfish and stickleback show that nerves from the myosepta enter the myotomes to form an elaborate network between the muscle fibres. Fig. 44 shows

some of these interlacing nerves in a stickleback myotome. Only a small proportion of the nerves in the section could be brought into focus at one time, but it can be seen that nerves are present at all depths of the myotome, and frequently a trio of closely adjacent nerves is found encircling the shaft of a muscle fibre (fig. 45).

In sections of the goldfish and stickleback examined with the electron microscope, it is often found that the fine myelinated nerves between muscle fibres are in groups of three, but not more than a single ending has yet been seen on a muscle fibre. No ending has been found in the vicinity of a myotendinous junction, the structure of which, in the goldfish, is shown in fig. 46. Mitochondria are numerous in the terminal zone of sarcoplasm, and sarcolemmal clefts are few and shallow. A few myofibrils can be seen pursuing a solitary course between the groups of mitochondria in the terminal zone of sarcoplasm.

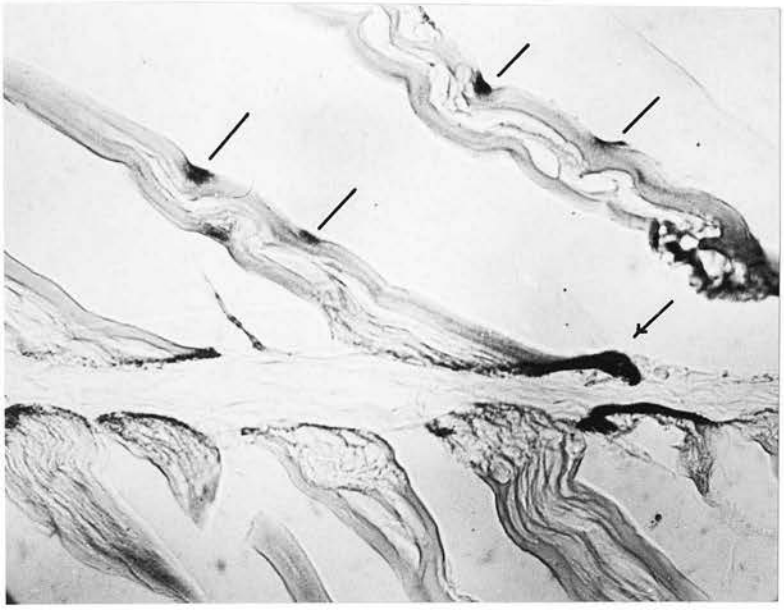


Fig. 28 : Dogfish (acetylthiocholine) - muscle fibres meeting myoseptum; the arrow points to myotendinous staining; the lines indicate motor endplate sites. (x 110)

Fig. 29 : Dogfish - (acetylthiocholine) showing two closely adjacent sites of cholinesterase concentration. (x 150)

Fig. 30 : Dogfish (acetylthiocholine) - superficial muscle; the patches of enzyme activity are scattered. (x 180)

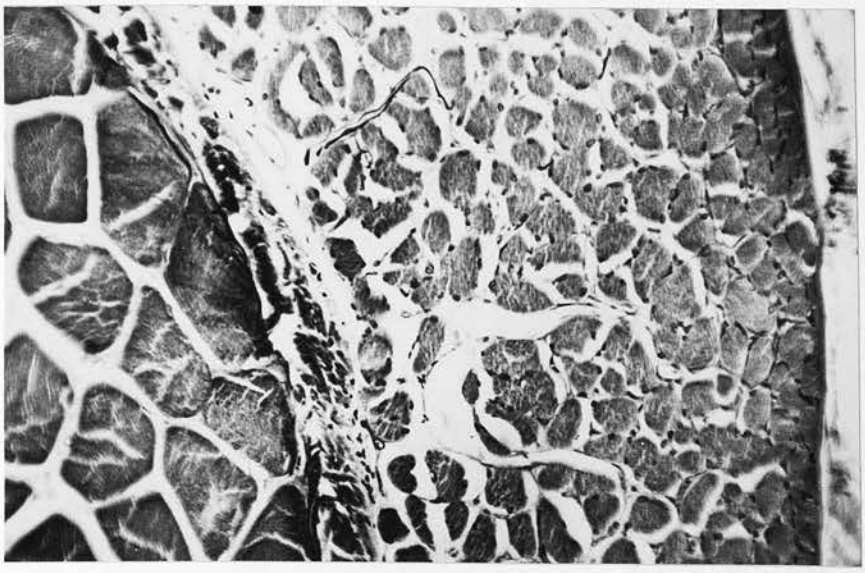


Fig. 31 : Dogfish (silver) - the differing calibres of the superficial and deep muscle is evident. (x 320)

Fig. 32 : Dogfish (silver) - superficial muscle fibres cut longitudinally; nerves can be seen at varying points along their shafts. (x 240)

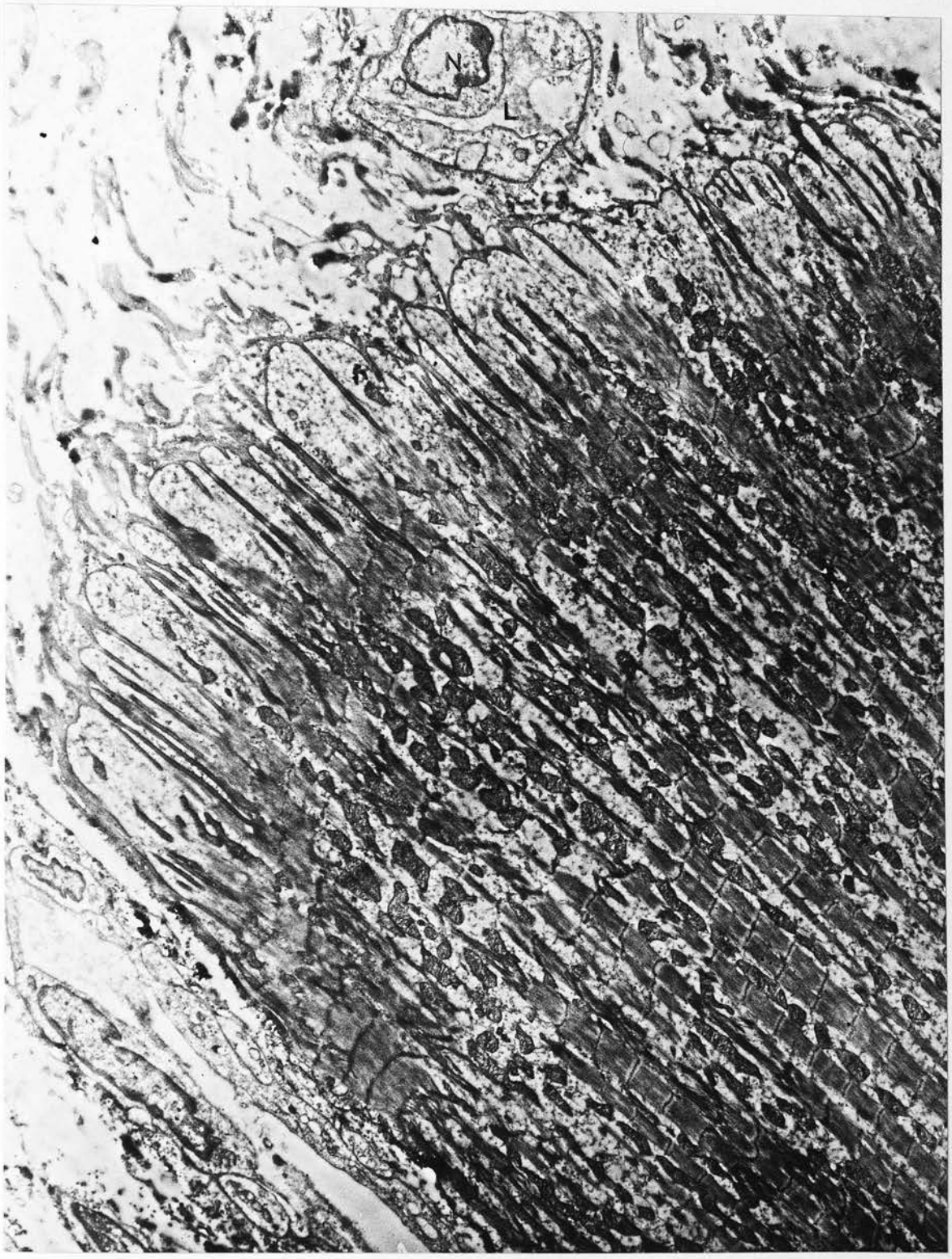


Fig. 33 : Dogfish (electron micrograph) - muscle-tendon junction; a capillary (lumen = L; nucleus = N) can be seen in the myoseptum. (x 3,000)

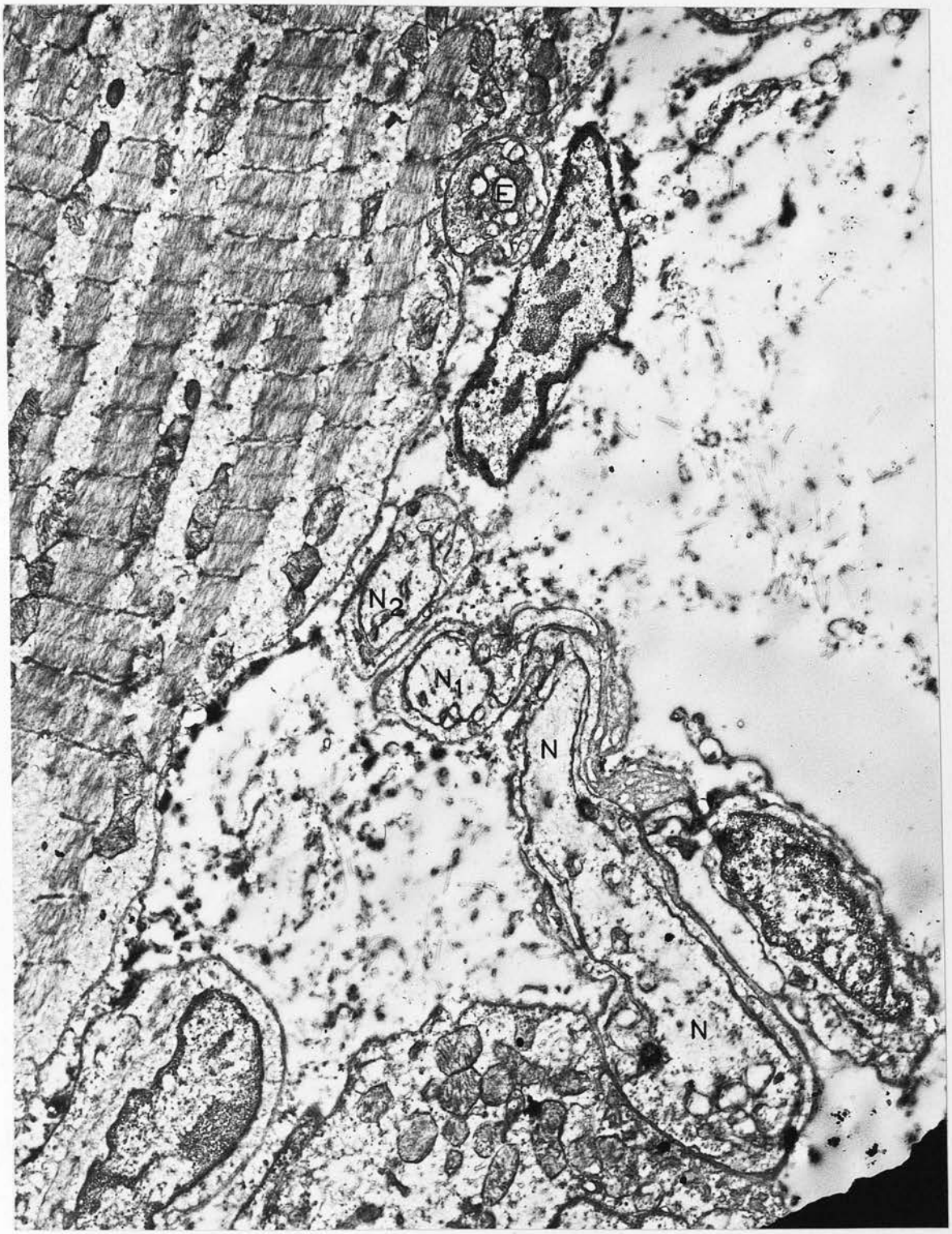


Fig. 34 : Dogfish (electron micrograph) - in the upper part of the plate, an endplate (E) can be seen set into the side of a muscle fibre; the lower half of the plate shows nerves (N, N_1, N_2), the nerve N terminating on another muscle fibre. (x 10,000).

Fig. 35 : Dogfish (electron micrograph) - the endplate (E) of fig. 34 at higher magnification. (x 60,000). G = groove of subneural apparatus.

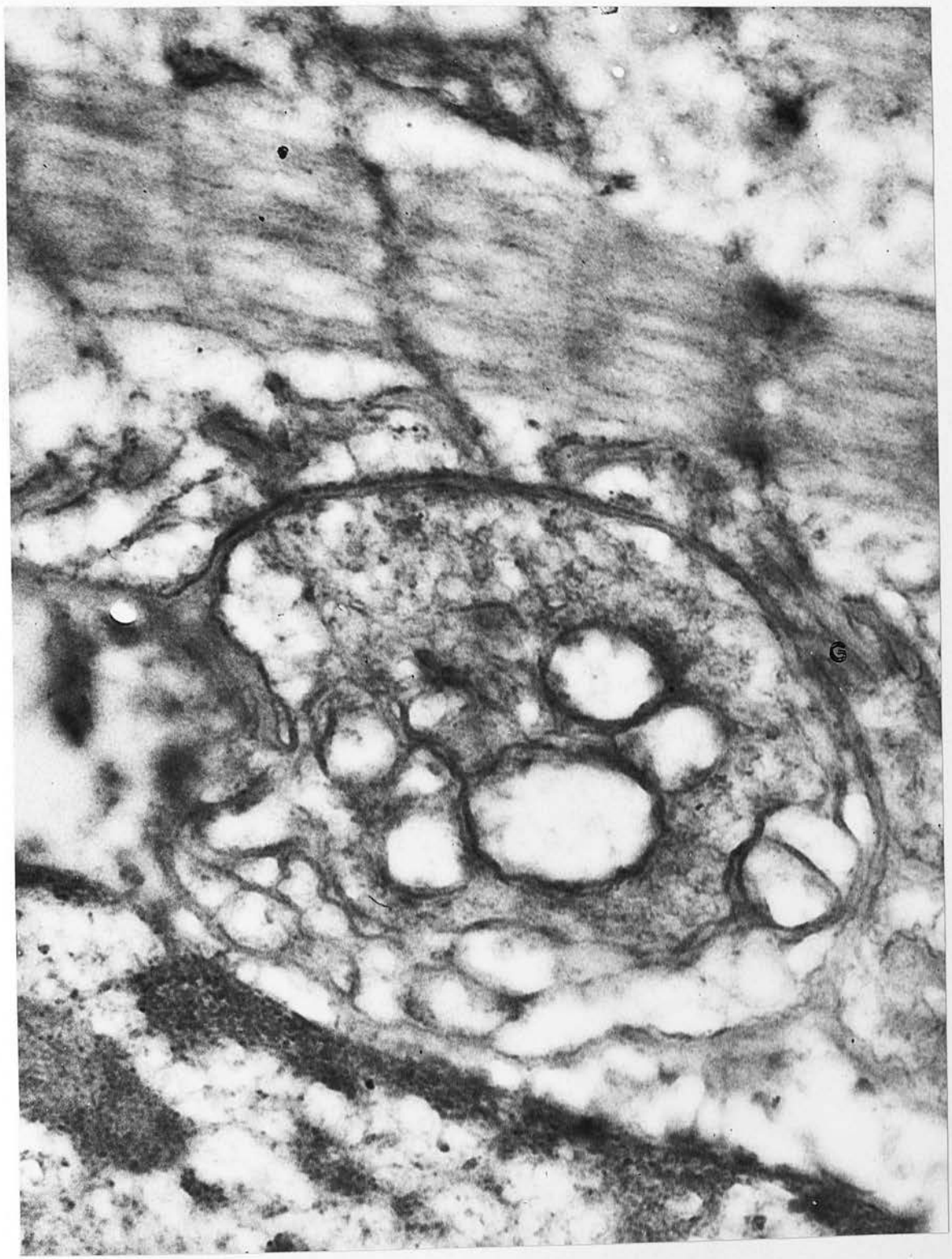


Fig. 36 : Dogfish (electron micrograph) - showing the neuromuscular junction from the lower part of fig. 34 at higher magnification. Arrows indicate points of reflection of Schwann cell sheath. G = grooves of subneural apparatus. (x 40,000)

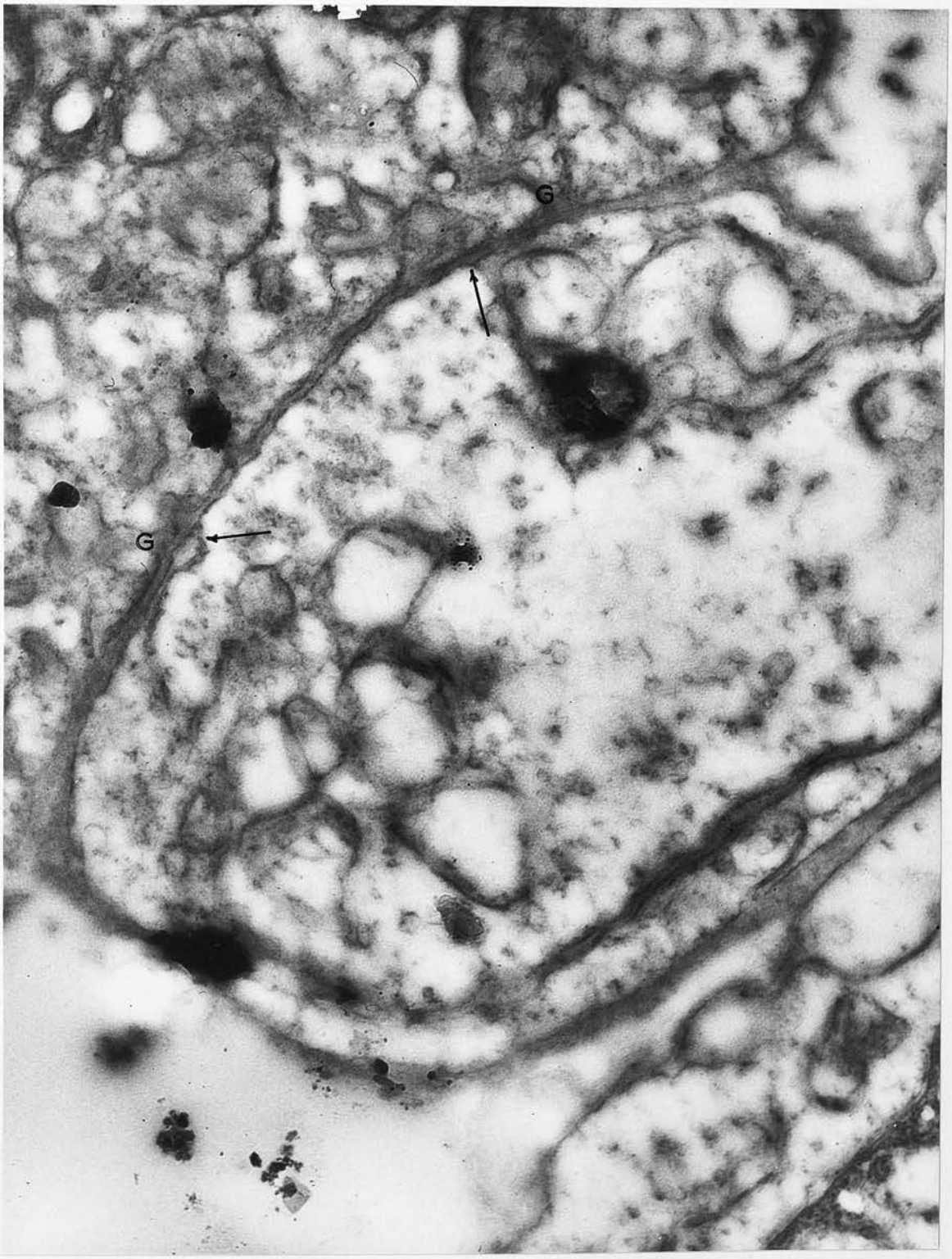


Fig. 37

(x 100)

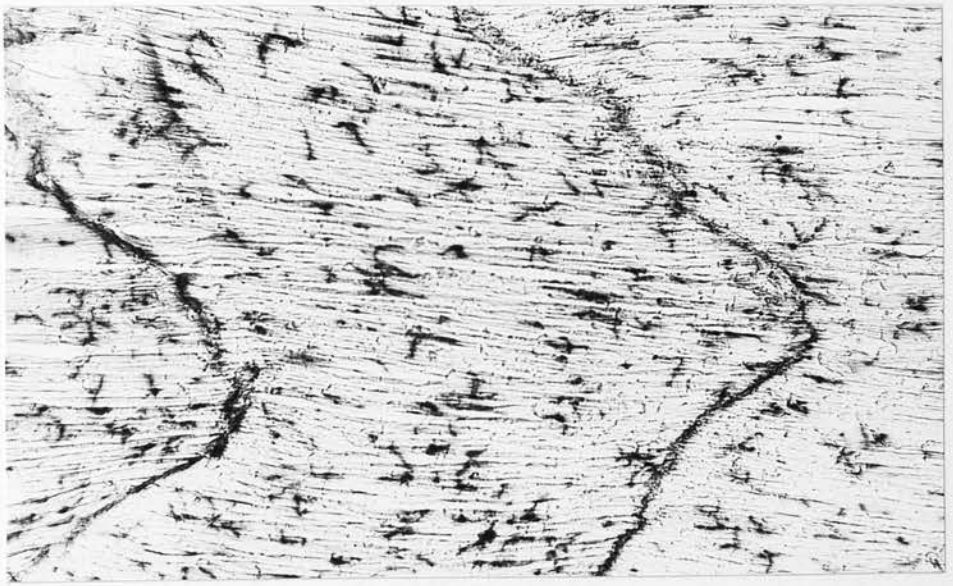
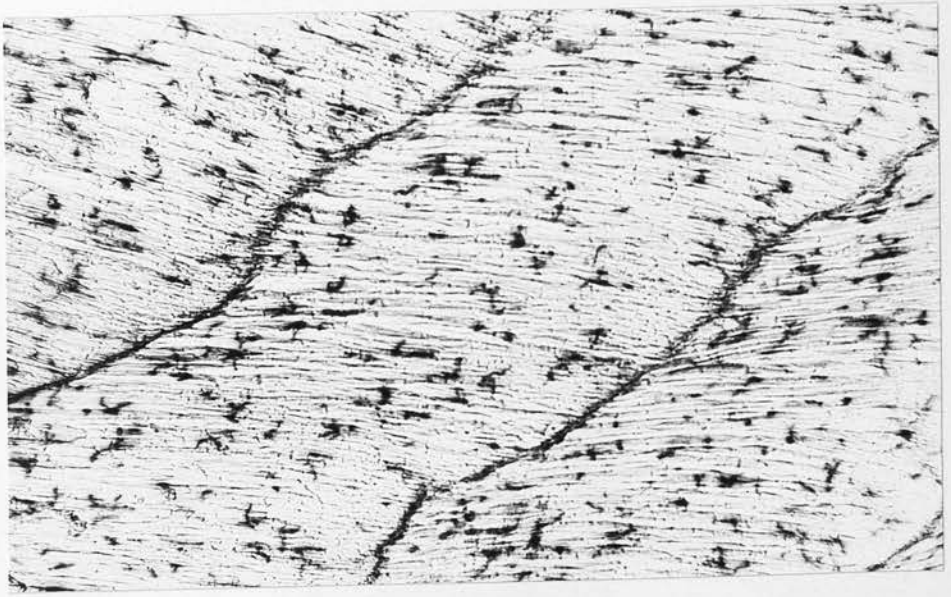


Fig. 38

(x 100)



Goldfish (azo-dye) - cholinesterase distribution in myotomes.

Fig. 39 : Loach (azo-dye) (x 400)

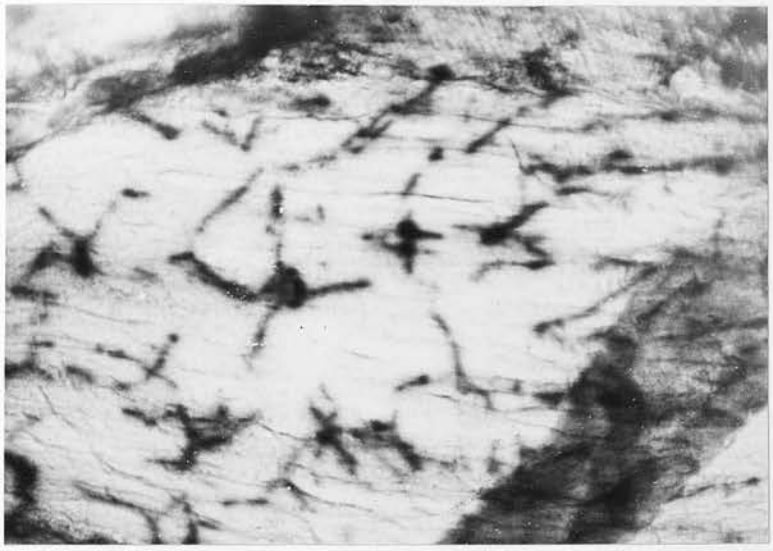


Fig. 40 : Trout (azo-dye) (x 400)

Fig. 41 : Salmon (azo-dye) (x 400)

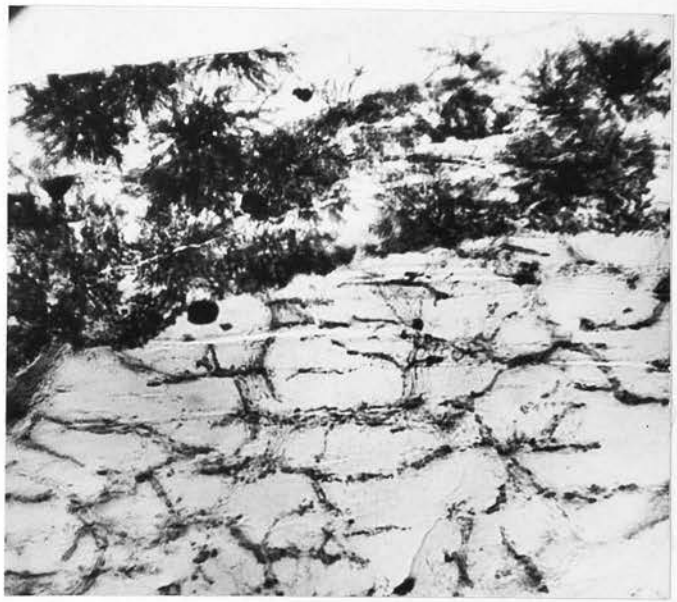


Fig. 42 : Minnow (acetylthiocholine)
(x 360)

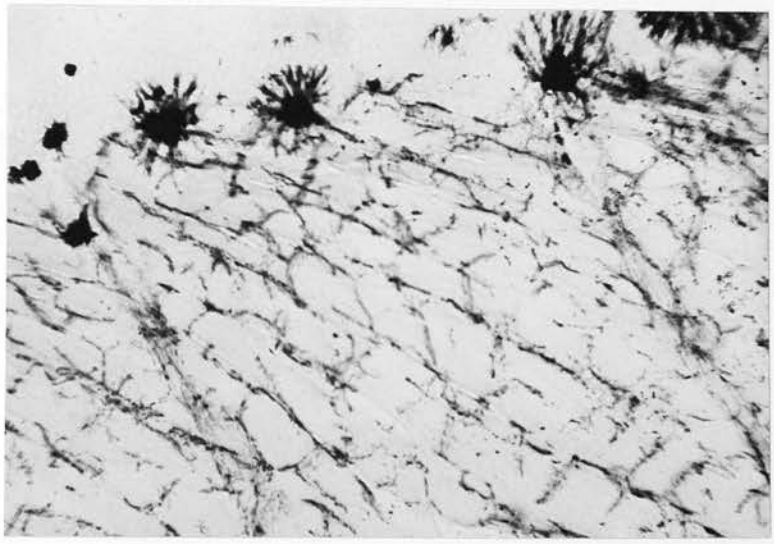


Fig. 43 : Stickleback (acetylthiocholine)
(x 360)

Fig. 44

(x 360)

Fig. 45

(x 740)

Stickleback (silver)

Fig. 46 : Goldfish (electron micrograph) - segmental muscle fibre meeting myoseptum. (x 5,000)

Amphibia

Cholinesterase studies and silver staining were carried out on three Urodeles and the larvae of two Anura. The Urodeles were salamander, newt, and pleurodele; and the larval Anura those of the frog, and Xenopus. Electron microscopy was confined to Xenopus larvae.

Whole mounts of a salamander and a Xenopus larva, stained for cholinesterase, are shown in figs. 47 and 48; the salamander is stained with the acetylthiocholine method, and the Xenopus larva using the azo-dye procedure. The enzyme activity is seen to be confined to the regions of the myosepta. An apparently single line of dye is present in each myoseptum, but in the frozen section of a salamander in fig. 49, it can be seen that in places this single line splits into two diverging lines. This occurs at the mid-dorsal surface in Urodeles, and in the region of the lateral line in the terminal myotomes of Anuran larvae. The two diverging lines follow the separating margins of the adjacent myotomes. At higher magnification, it can be seen that there is a cone of dye over the tip of every muscle fibre (fig. 50), and when single muscle fibres are isolated, either by agitating frozen sections gently in water to reduce the number of fibres present (fig. 51), or by teasing individual fibres from pieces of stained whole mounts (fig. 52), it can be shown that dye is present at both ends of every muscle fibre.

The nerves in silver-stained sections are seen to be almost exclusively within the myosepta. A plexus is formed in the fibrous tissue of a myoseptum by a segmental nerve, and from this plexus nerves run towards the tips of the muscle fibres (figs. 53 and 54). A number of nerves from the plexus may run direct to the tip of a single muscle fibre, or a nerve may pass over several fibres giving branches to each of them. On reaching the end of a muscle fibre, a nerve divides further, the branches ramifying over the sarcolemma in a distribution which corresponds with the extent of the zone of cholinesterase activity (figs. 56 and 57). It is rare for a nerve to penetrate into the depths of a myotome, but occasionally in Xenopus larvae a nerve could be followed for some distance alongside a muscle fibre (fig. 55).

Electron microscopy of Xenopus larvae shows the close relationship between the fine terminal nerve fibres and the sarcolemma covering the ends of the muscle fibres. As in other species, the myotendinous junction in Xenopus is characterised by a series of clefts which pass up through the zone of sarcoplasm at the end of the muscle fibre. The myofibrils are attached to the inner surface of the sarcolemma passing up into the clefts, and collagen fibrils emerge from the clefts to form tendon.

As a nerve approaches the sarcolemma it is invested by a thin sheath of Schwann cell cytoplasm, but this is reflected as the axon comes into contact with the sarcolemma. The

unmyelinated nerve fibres are applied to the sarcolemma between the clefts, or at the side of the muscle fibre close to its termination. Fig. 58 shows a nerve ending at the end of a Xenopus muscle fibre. Myofibrils (M) can be seen running down to meet the clefts (C), from which collagen fibrils are emerging. Large, pleomorphic muscle mitochondria (M_1) are scattered through the zone of sarcoplasm beyond the myofibrils, and contrast with the smaller, more dense nerve mitochondria in the ending. A schwann cell nucleus (N) is present. A smaller ending (E_1) can be seen near the edge of the plate.

Fig. 59 shows a different ending. Again there is no deep penetration of the muscle fibre by the ending, but shallow pockets in the subjacent sarcolemma are present. The complex subneural apparatus of higher vertebrate neuromuscular junctions is not found in Xenopus segmental muscle.

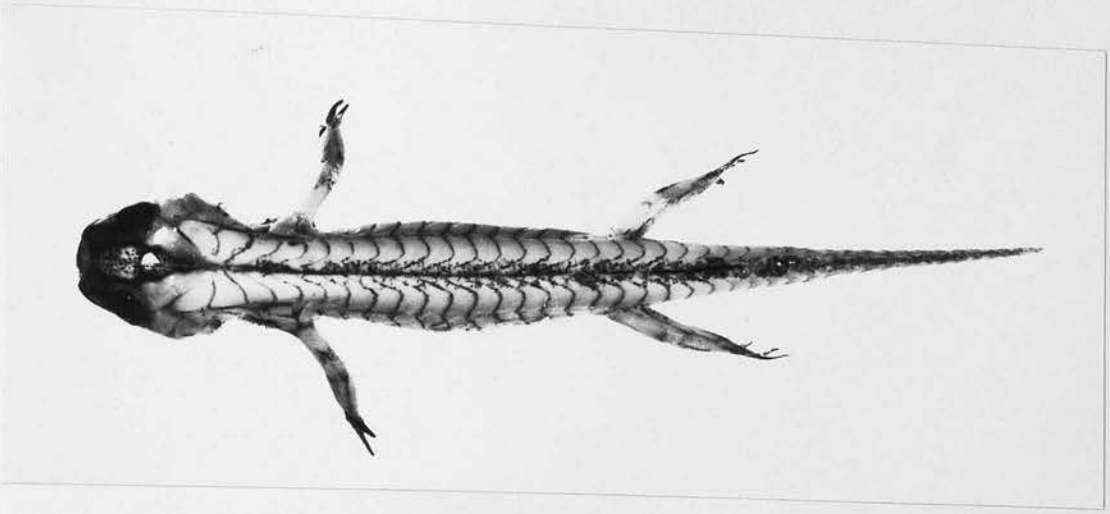


Fig. 47 : Salamander (acetylthiocholine) - the dye is confined to the lines of the myosepta. (x 4)

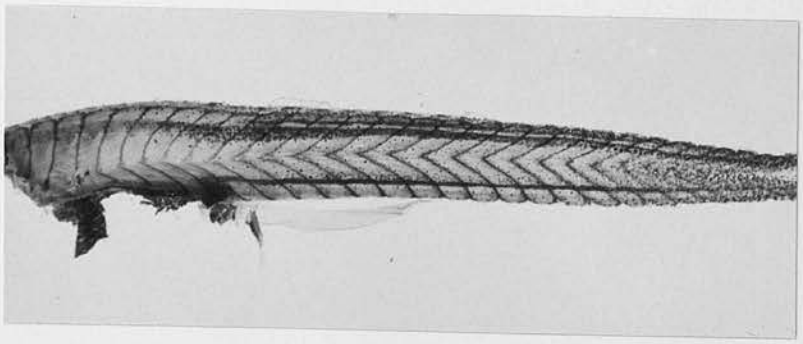


Fig. 48 : Xenopus larva (azo-dye) - part of the tail of a whole mount; the V-shaped myosepta are shown up by the dye. (x 6)

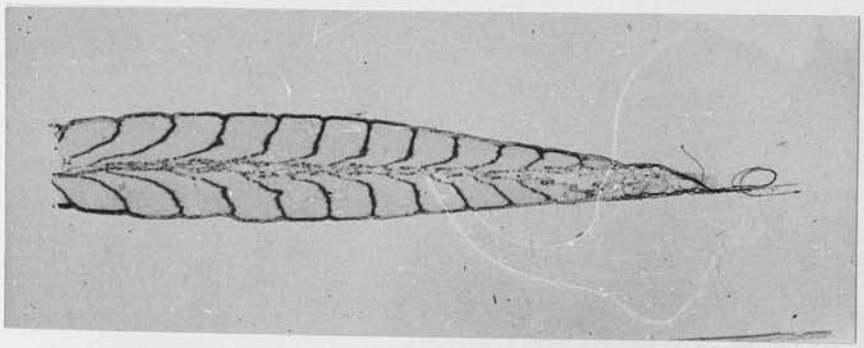


Fig. 49 : Salamander (azo-dye) - frozen section cut in horizontal plane; the dye appears along the lines of the myosepta and in the skin. (x 6)

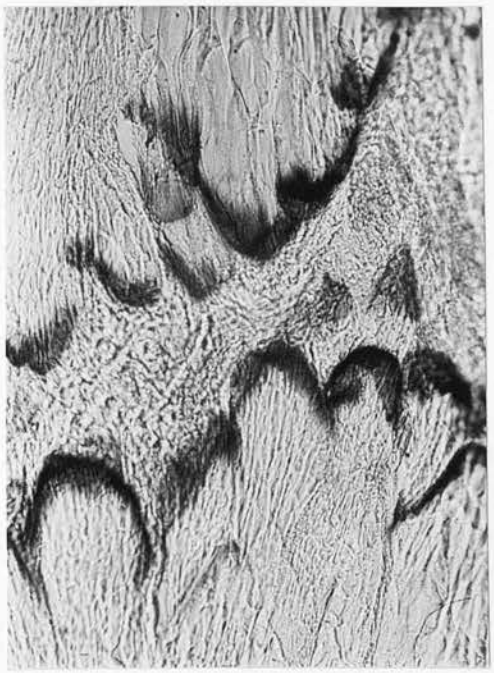


Fig. 50 : Pleurodele (azo-dye) - frozen section showing myoseptal region; the dye is confined to the tips of the muscle fibres. (x 110)

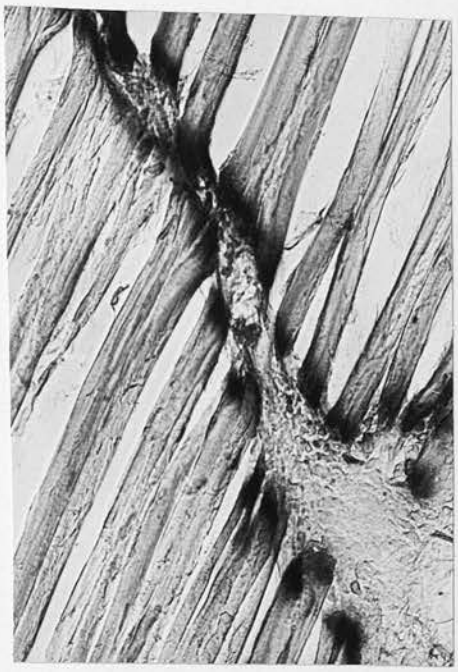


Fig. 51 : Salamander (azo-dye) - frozen section in which the fibres have been thinned out; every muscle fibre is capped with dye. (x 330)

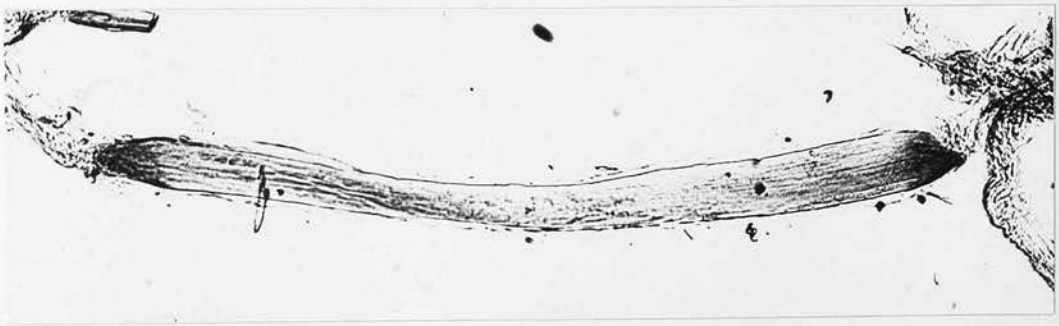


Fig. 52 : Newt (azo-dye) - single fibre isolated from stained whole mount; dye is present at both ends of the fibre. (x 270)

Fig. 53 : Salamander (silver) - showing branching of the myoseptal plexus of nerves. (x 510)

Fig. 54 : Xenopus larva (silver) - showing branching of the myoseptal plexus of nerves. (x 510)

Fig. 55 : Xenopus larva (silver) - a nerve can be seen supplying the tip of a muscle fibre with branches and then running alongside the fibre for some distance to supply it with further small filaments. (x 510)

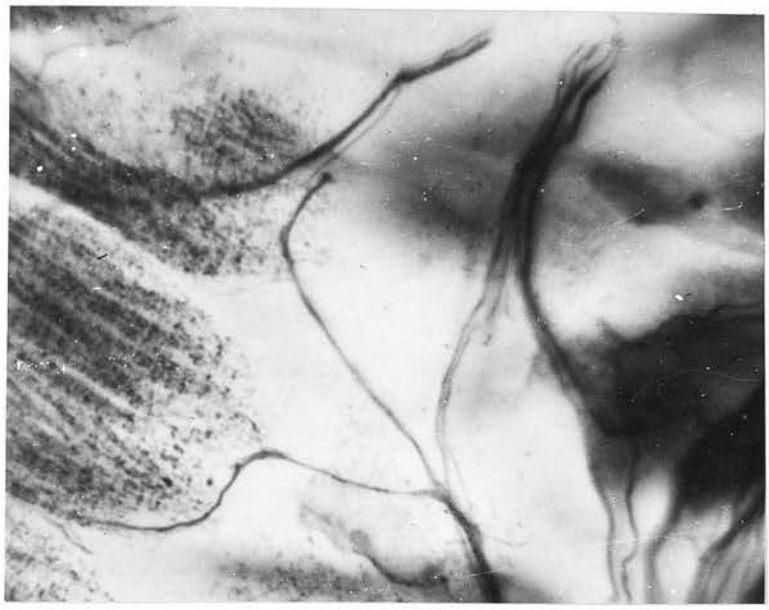


Fig. 56 : Salamander (silver) - a nerve in the myoseptal plexus divides to supply two adjacent muscle fibres. (x 1480)

Fig. 57 : Newt (silver) - branches from the myoseptal plexus envoloping the end of a muscle fibre. (x 1640)

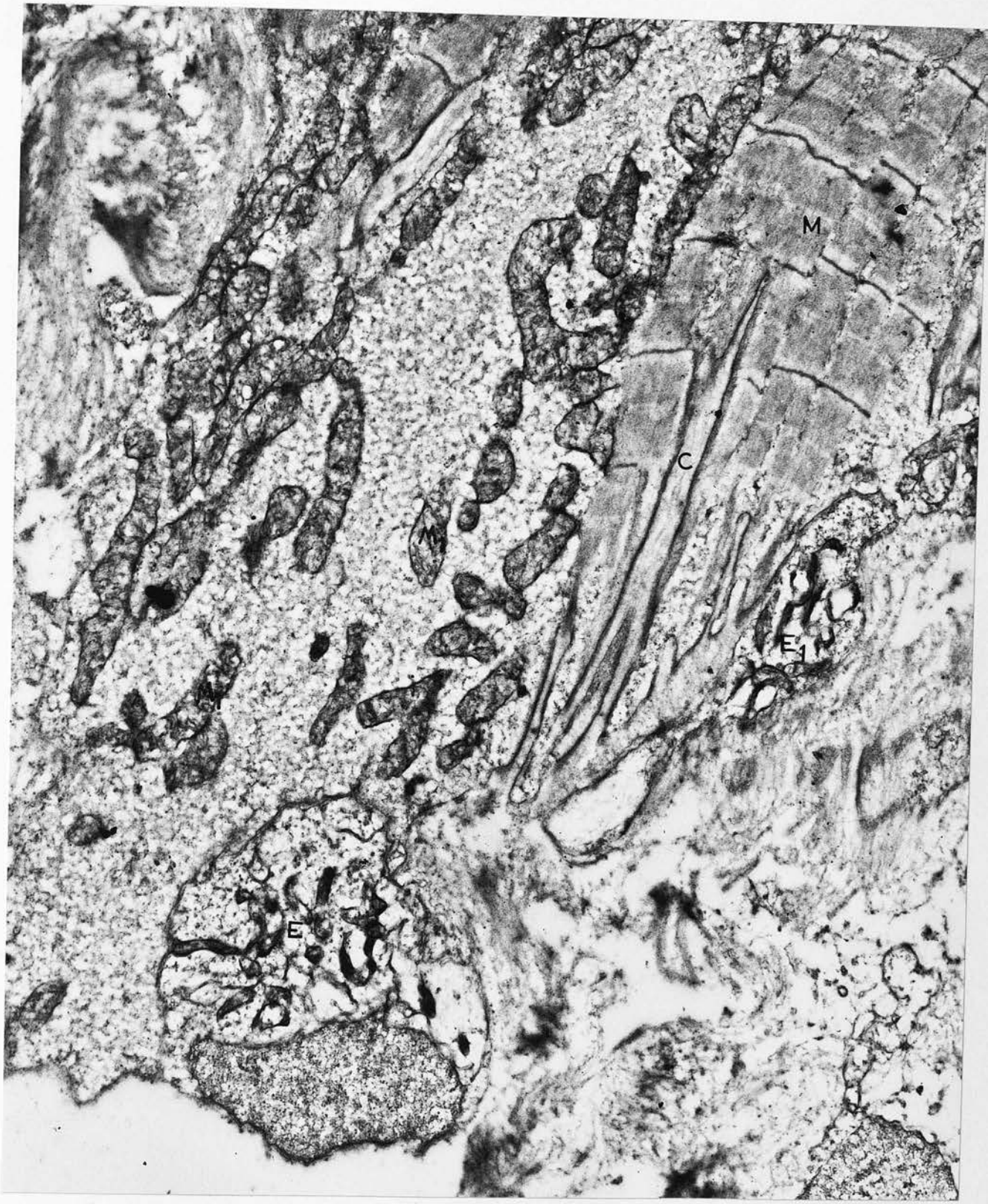


Fig. 58 : Xenopus larva (electron micrograph) - end of a segmental muscle fibre, with two nerve endings (E and E_1) closely applied to the sarcolemma. C = cleft of myotendinous region; M = muscle myofibrils. (x 14,000)

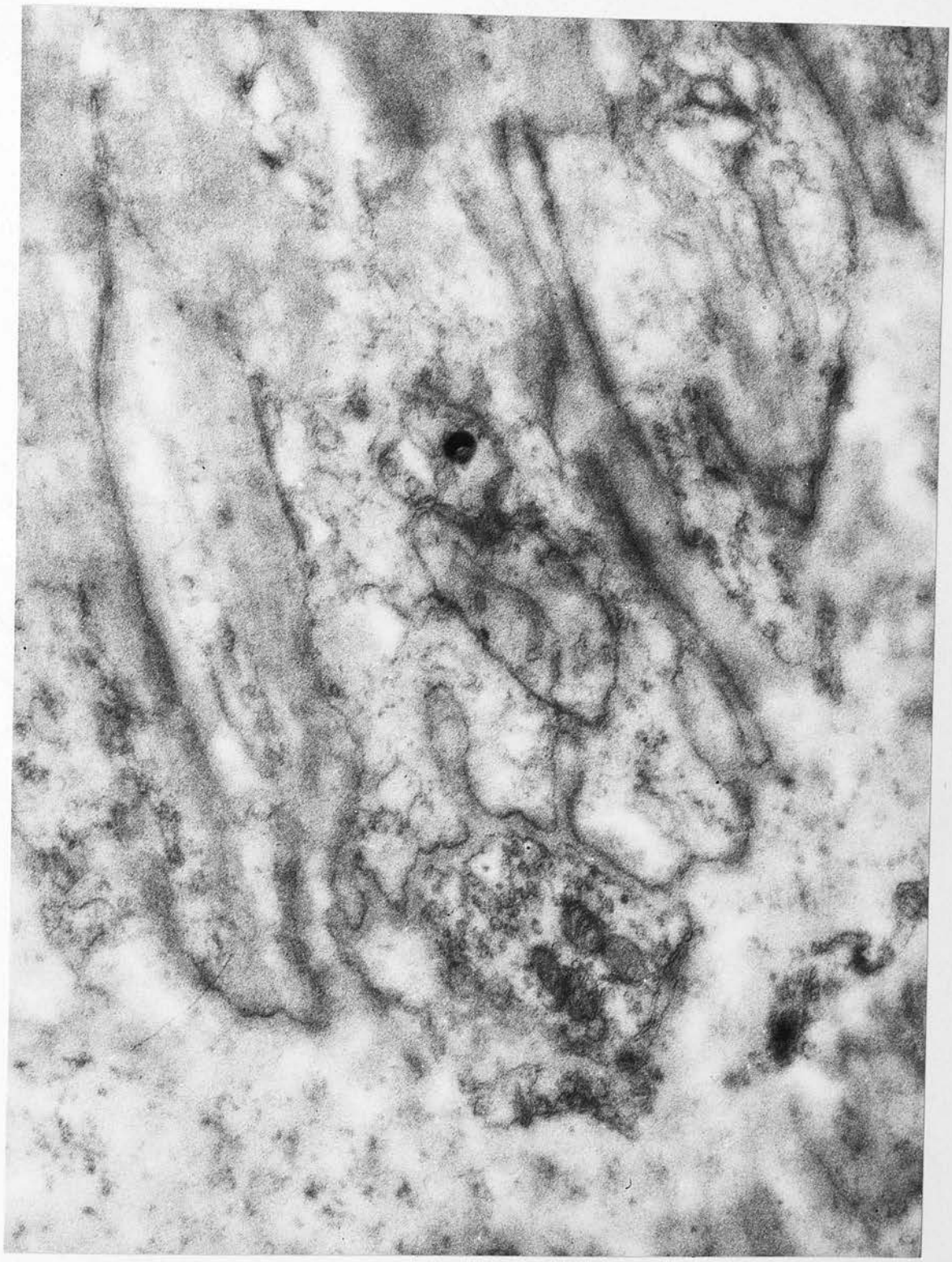


Fig. 59 : Xenopus larva (electron micrograph) - end of a segmental muscle fibre with a nerve ending; deep to the ending, grooves of the subneural apparatus can be seen. (x 21,000)

Reptilia

Two reptiles were studied - the grass snake, and the green lizard. They will be described separately.

Snake :

The true segmental musculature of the snake lies caudal to the anal orifice. In fact, no difference has been found in the innervation of pre- and post-anal musculature.

Cholinesterase-stained sections show that the motor endplates are grouped together in fairly broad bands that extend down the middle of the intercostal spaces (fig. 60). The endplates are clearly stained, but structural detail is not evident; this may be the result of overlong incubation, since the preparations were intended primarily as whole mounts. The endplate in fig. 61 has been photographed from the preparation in fig. 60, and it can be seen that the outline of the zone of cholinesterase is fairly sharp, indicating that relatively little diffusion has occurred. The outline is also fairly regular, as was the case with all the other snake endplates examined, and this is in marked contrast to the irregular configuration of the lizard endplate in fig. 66. Cholinesterase also appears near the ribs, at the sites of the muscle-tendon junctions, and this is illustrated in fig. 62.

Snake muscle fibres were examined in thin sections with the electron microscope, and a view of a muscle fibre is shown in fig. 63. A striking feature of this section is the large

proportion of sarcoplasm in relation to the myofibrils, and this was seen quite frequently in snake muscle although the majority of sections showed many more myofibrils. It will be noted, too, that mitochondria are plentiful. The sarcolemma on both sides of the muscle fibre is crenated, the grooves being related to the Z-bands of the underlying myofibrils.

A motor endplate on a snake muscle fibre is shown in fig. 64. Its main features are the extent of the ending, and the complexity of the subneural apparatus. The sarcolemmal folds constituting this subneural apparatus are longer than any seen in Xenopus, and show frequent branching.

Lizard :

The tail muscle was mainly studied, but the opportunity was taken also to observe the distribution of cholinesterase in the intercostal muscle, and the appearances of the two were identical.

Motor endplates in the lizard tail muscle stained well at pH 5 or pH 6. The duration of incubation appeared of less consequence than in any other segmental muscle, and up to two hours fixation did not appear to interfere with the quality of staining. Even after seven hours incubation, the details of endplate structure could be seen clearly, though a certain amount of diffusion had occurred. The preparations shown in figs. 65 and 66 were, however, incubated for two

hours only.

In contrast to the orderly band of endplates in the snake intercostal muscle, both the intercostal and tail muscle of the lizard show no organisation of the endplates into bands. Instead, the endplates are quite irregularly scattered through the sections, some being near the middle of the muscle fibres, and others close to the fibre junction with tendon (fig. 65 - arrows). Here, too, there is a cholinesterase reaction, but there is no difficulty in distinguishing it from an adjacent endplate with its distinctive structure (fig. 66).

An electron micrograph of an endplate from the tail muscle of the lizard is shown in fig. 67. As in the snake, the subneural apparatus is extensive and elaborate, with narrow, deep and tortuous invaginations showing frequent branching.

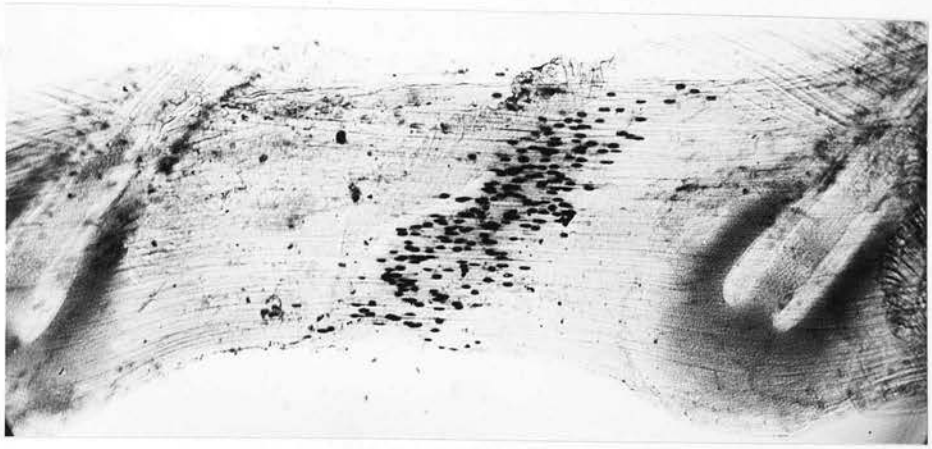


Fig. 60 : Snake (acetylthiocholine) - whole mount of intercostal muscle; the endplates appear as a broad band down the middle of the space. (x 40)

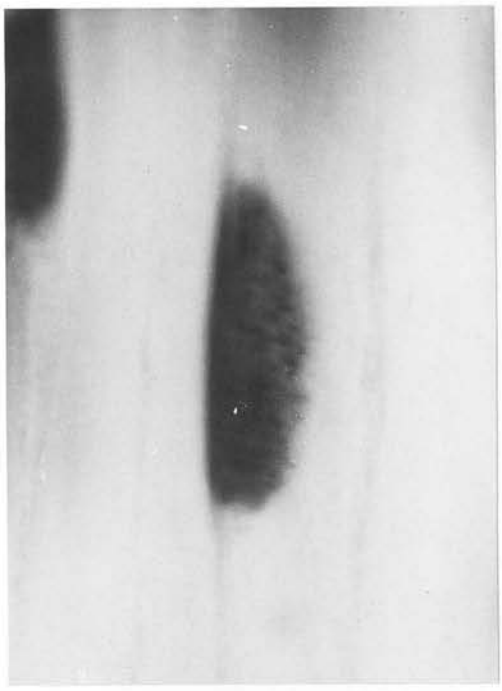


Fig. 61 : Snake (acetylthiocholine) - endplate from preparation in fig. 60. (x 450)

Fig. 62 : Snake (acetylthiocholine) - myotendinous reaction from preparation in fig. 60. (x 410)

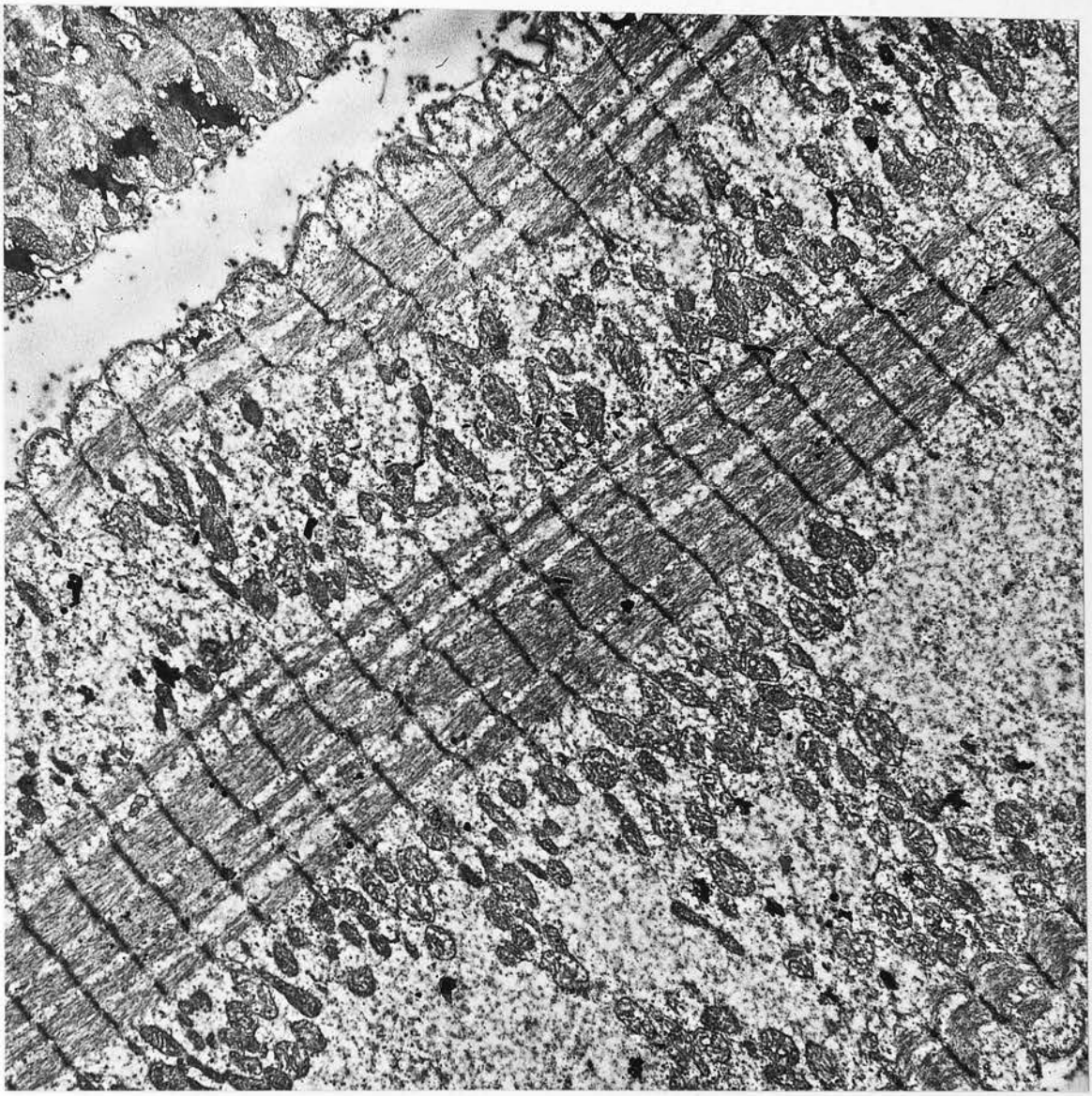


Fig. 63 : Snake (electron micrograph) - intercostal muscle fibre, showing a particularly high sarcoplasm content. (x 8,000)

Fig. 64 : Snake (electron micrograph) - motor endplate from intercostal muscle; the arrows indicate the limits of the subneural apparatus. (x 10,000)

Fig. 65 : Lizard (acetylthiocholine) - showing the scatter of endplates in the tail muscle; some of the endplates are indicated by arrows.
A prominent myotendinous reaction is present.
(x 40)

Fig. 66 : Lizard (acetylthiocholine) - showing an endplate in close proximity to the end of a muscle fibre which also gives a myotendinous response.
(x 500).

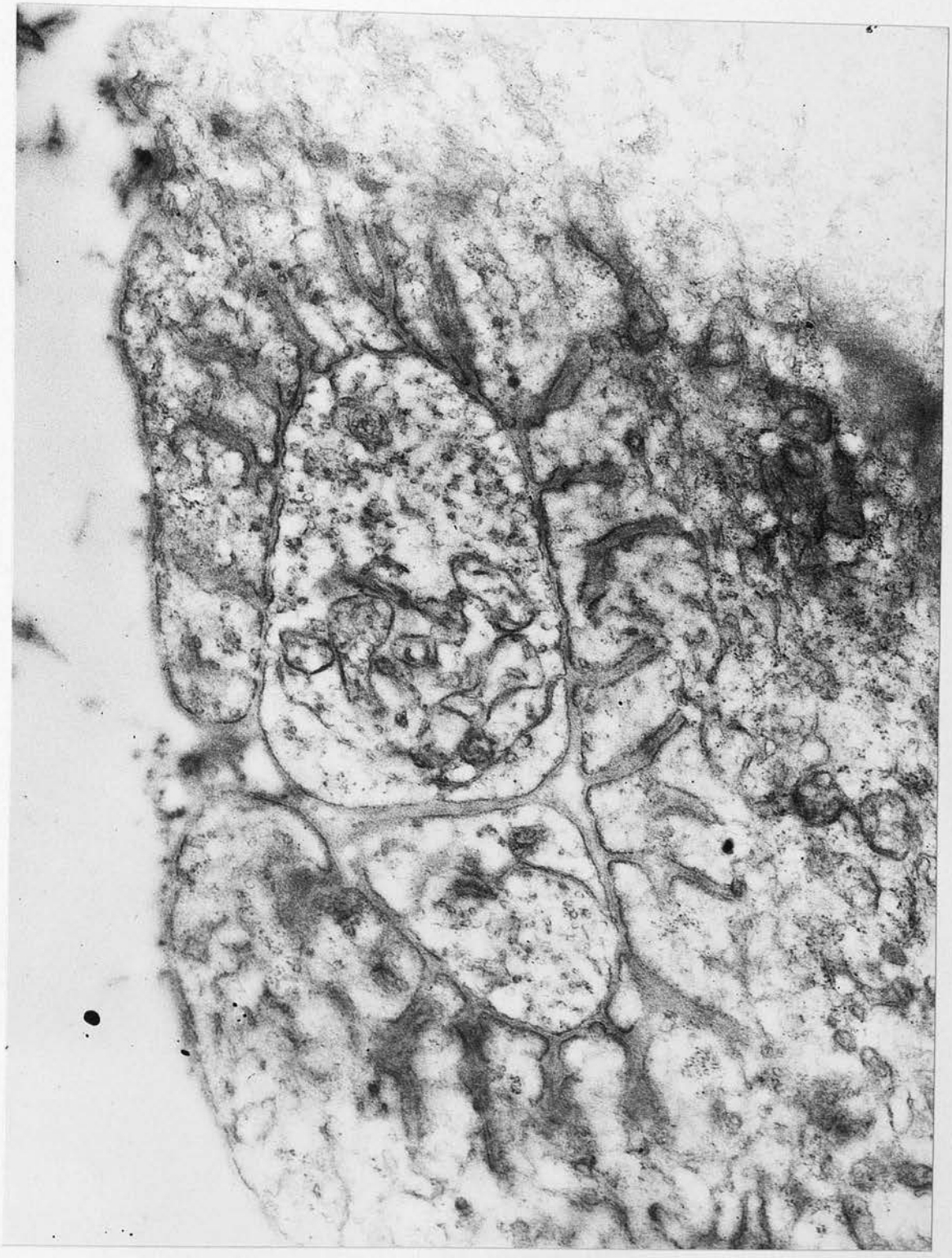


Fig. 67 : Lizard (electron micrograph) - motor endplate from tail muscle. (x 37,000).

2. Avian Muscle.

Ginsborg (1959) reported from electrophysiological studies that muscle fibres of the chick are innervated in one of two different ways. One type of fibre is supplied by a single axon and has a focal endplate. The other type is supplied by a number of axons and has neuromuscular junctions distributed at many points along the fibre. The attempt was therefore made to demonstrate these two types of innervation by histochemical methods.

Methods

The muscles principally studied were the anterior and posterior latissimi dorsi of the chick, since these had been shown by Ginsborg (1959) to contain at least very high proportions of the multiply-innervated and focally-innervated fibres respectively (for convenience the fibres with many neuromuscular junctions are referred to as multiply-innervated). Other chick muscles investigated were the semispinalis cervicis, biventer cervicis, lateral and medial gastrocnemii, sartorius, extensor digiti III and IV, and extensor metacarpi radialis profundus. The latissimus dorsi of the pigeon was also examined.

The object of the investigation was to display neuromuscular junctions by staining sites of cholinesterase concentration on the muscle fibres; and to determine the optimum conditions for staining in the bird muscles, a series of preliminary studies was made using the acetylthiocholine

technique. Some of the results of this study are referred to in the section of the discussion dealing with the histochemistry of cholinesterase.

The muscles were taken from birds whose ages ranged from two to sixteen weeks. The birds were killed with a lethal dose of 9% sodium phenobarbitone administered intravenously, and after perfusion through the left ventricle with 10% formalin, the muscles to be used were dissected free. Fixation was continued by immersion of the muscles in 10% formalin till a total period of one hour had been reached. They were then stained as whole mounts, or in small bundles of fibres, or as frozen sections. Both the acetylthiocholine and the azo-dye techniques were employed; the former gave better contrast in whole mounts and frozen sections, but less diffuse staining was obtained from the azo-dye procedure and this was therefore preferred where isolation of individual fibres was the ultimate aim. The incubating solution was prepared to pH 8 for both muscles and for both techniques.

When material was incubated for 30 minutes in 10^{-5} M. eserine sulphate before immersion in the incubating medium, no staining resulted. Further characterisation of the cholinesterase was not undertaken.

Results

Differences in appearance between the anterior and posterior latissimi dorsi were obvious on examination of the surface of stained whole muscles, or of frozen sections.

In the case of the anterior, a multitude of small dots was scattered over the surface of the muscle, or throughout the section (fig. 69), whereas in the posterior, considerable areas were free from stain (fig. 70).

Single muscle fibres were isolated from small bundles stained with the azo-dye method. The large amount of tenacious connective tissue in the anterior latissimus dorsi made it difficult to isolate more than short lengths of fibres, but longer lengths were obtained from the posterior.

Examination of lengths of single fibres from the anterior latissimus dorsi showed that these fibres have many neuromuscular junctions situated at short intervals along their length (figs. 71 and 72). In fibres from the posterior latissimus dorsi, from which the longest segment of fibre obtained was 1.7 cm., only single endplates were found (fig. 73).

A characteristic difference in appearance was often seen between the patterns of cholinesterase distribution on the fibres from the anterior and posterior latissimi dorsi (figs. 74 and 75). The enzyme pattern on fibres from the anterior latissimus dorsi has an appearance resembling that displayed by Couteaux (1958) on slow fibres from the frog, whereas that from posterior latissimus dorsi fibres is more comparable with endplates in mammalian muscle (Couteaux, 1958).

Using similar methods, a number of other chick muscles, as well as the latissimus dorsi of the pigeon, were examined. The appearance of frozen sections of the following muscles :

sartorius, lateral and medial gastrocnemii, biventer cervicis, and semispinalis cervicis : suggested that these muscles contained both types of fibre. This was confirmed by examination of lengths of single muscle fibres isolated from stained bundles of each of the muscles. In two other muscles examined : extensor carpi lateralis profundus, and extensor digiti III and IV : no fibres with multiple neuromuscular junctions were found. In the latissimus dorsi of the pigeon, only fibres with many neuromuscular junctions were seen.

An estimate of the average distance between adjacent neuromuscular junctions on fibres isolated from the anterior latissimus dorsi was made, either by counting the number of junctions in measured lengths of isolated fibres, or from measurements of individual intervals at magnifications of 40 x to 100 x. The average distance varied between 225 μ in segments of muscle fibres taken from a two week old chick, to 790 μ in fibres from a fifteen week old chick. The results are shown in fig. 76 for three different sizes of muscle. Considerable variation in individual values for the distance between adjacent neuromuscular junctions was observed (fig. 77), but this may in part have been the result of mutilation of the sarcolemma, which often occurred in the process of separating fibres, with consequent loss of some of the junctions.

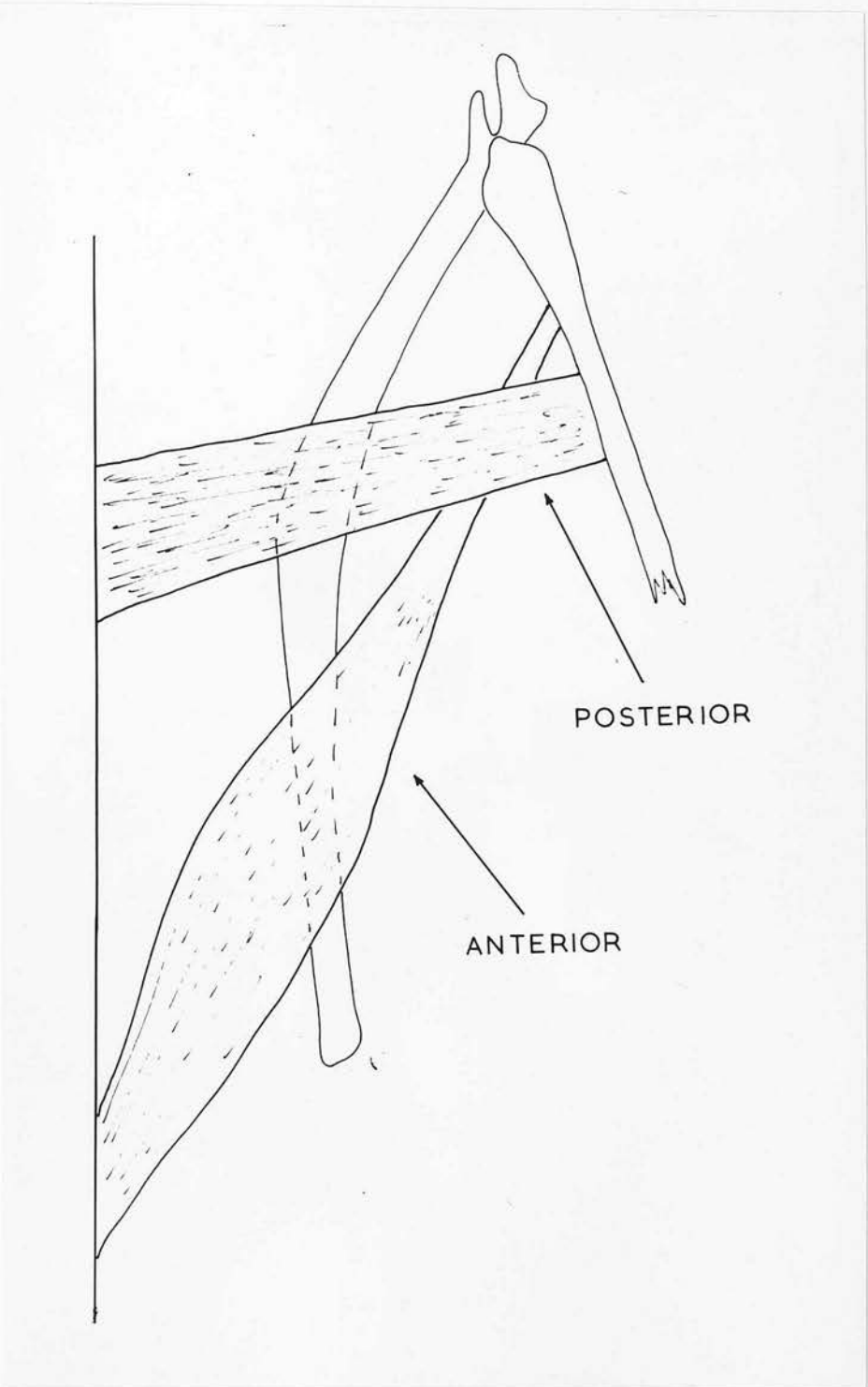


Fig. 68 : Diagram to show the relative positions of the anterior and posterior latissimus dorsi muscles in the chick.

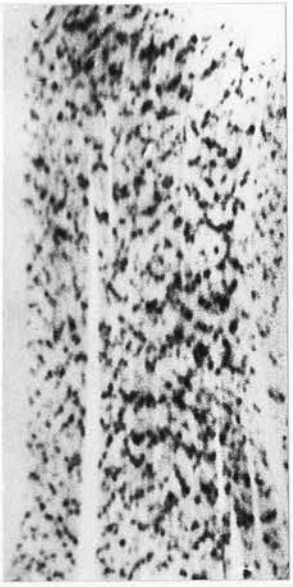


Fig. 69

(x 7.5)

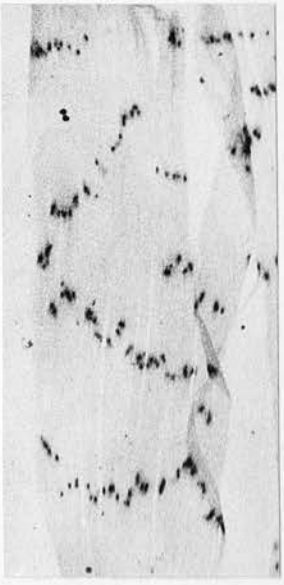


Fig. 70

(x 7.5)

Frozen sections (acetylthiocholine) - fig. 69 from the anterior latissimus dorsi of a six-week old chick; fig. 70 from the posterior latissimus dorsi of a ten-week old chick.

Fig. 71 : Segment of fibre from anterior latissimus dorsi of 16-week old chick. (x 30)

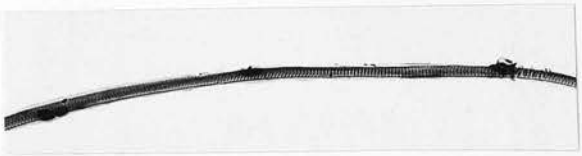


Fig. 72 : Segment of fibre from anterior latissimus dorsi of 2-week old chick. (x 135)

Fig. 73 : Segment of fibre from posterior latissimus dorsi of 6-week old chick. (x 60)

Fig. 74

(x 125)

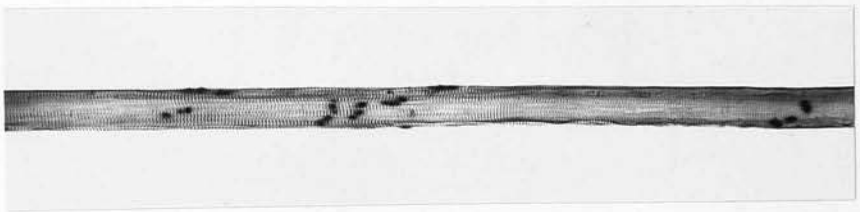
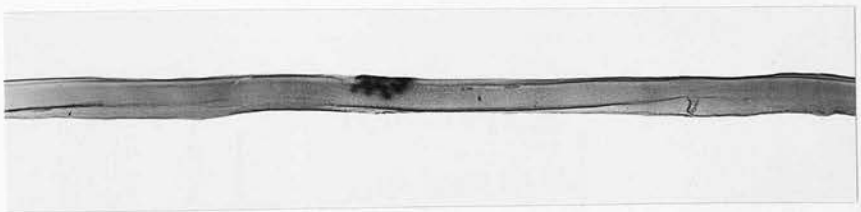


Fig. 75

(x 125)



Segments of fibres from anterior (fig. 74) and posterior (fig. 75) latissimus dorsi of a 15-week old chick; (azo-dye).

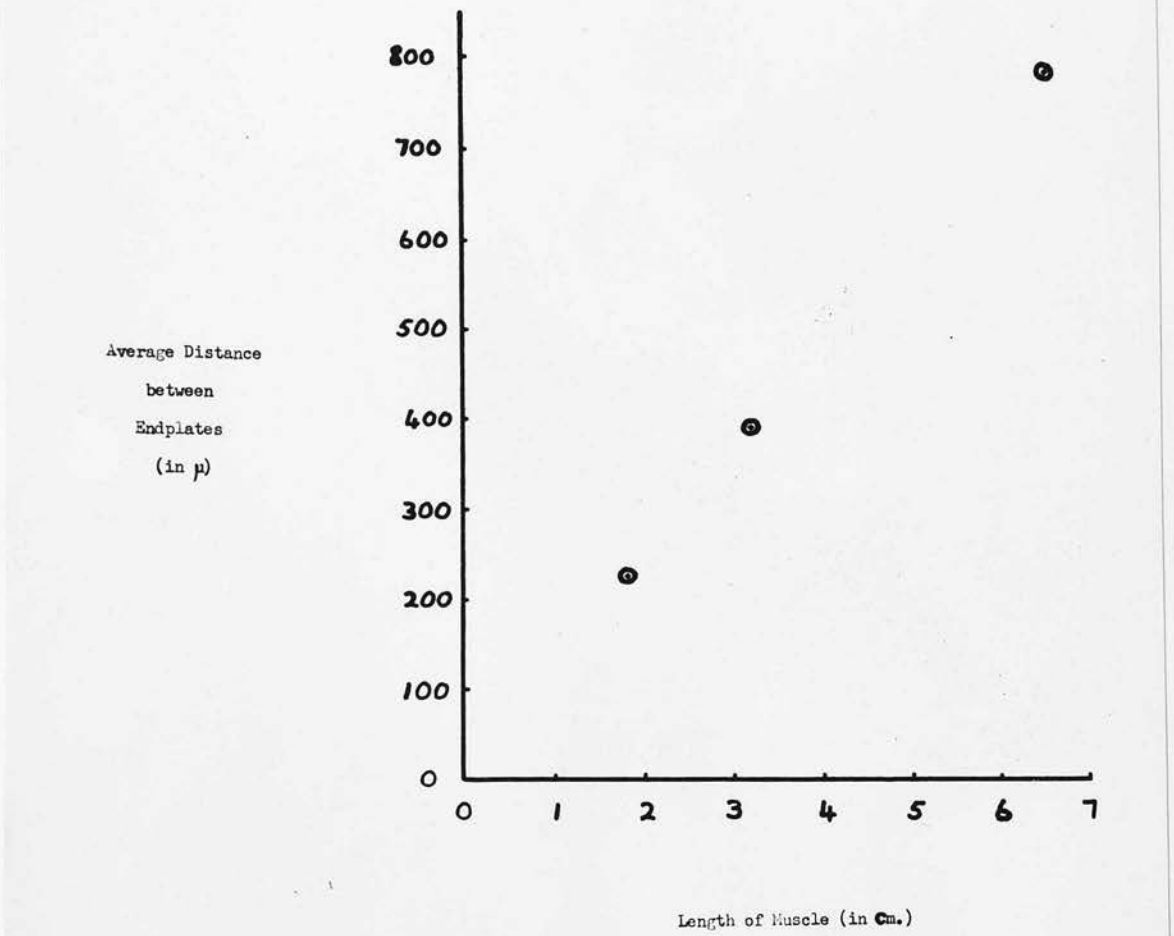


Fig. 76 : Relationship between average distance between adjacent endplates on fibres from three anterior latissimus dorsi muscles, and length of muscle.
Bird A : aged 2 weeks; weight 85 G.; number of intervals measured 97.
Bird B : aged 4 weeks; weight 590 G.; number of intervals measured 61.
Bird C : aged 15 weeks; weight 1,300 G.; number of intervals measured 72.

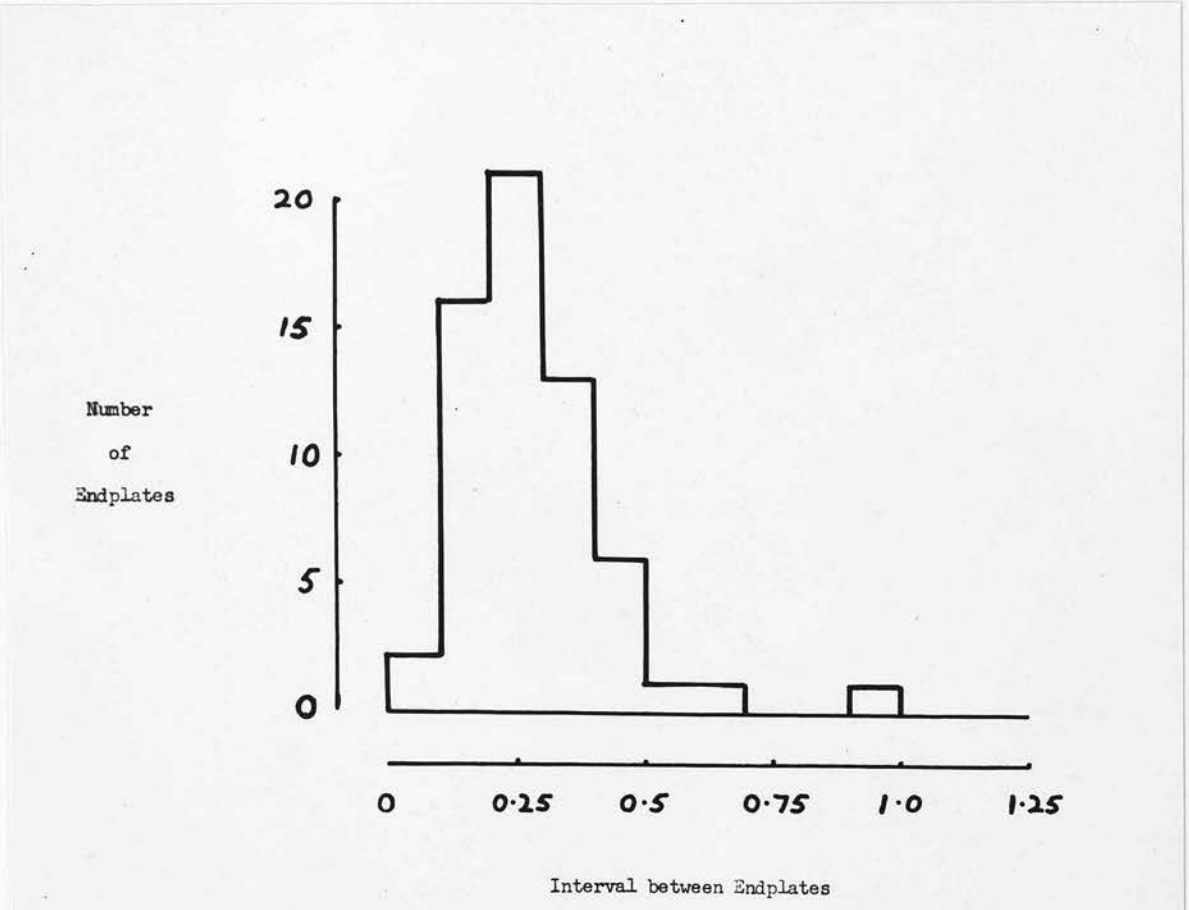


Fig. 77 : Variation of distance between adjacent neuromuscular junctions in fibres from anterior latissimus dorsi muscle of a four-week old chick. Interval between endplates in mm.

3. Mammalian Muscle.

A number of muscles from each of several species of mammal were stained as whole mounts using the acetylthiocholine technique, to display the distribution of motor endplates in the muscles as a whole. With this method, the surface only of the muscle was stained, but in the few cases in which the interior of the muscle at various depths was also investigated, it was found that the internal distribution of endplates was the same as that on the surface; the latter could therefore be taken as representative of the whole muscle.

In preliminary experiments with this technique on mammalian muscle, it was found that there is considerable latitude in the range of the conditions under which these will stain. Thus, in material from the diaphragm of the rat, incubated at pH 4, 5, 6, 7, staining was adequate at all pH values, though more marked and with minimal diffusion at pH 5. This pH was therefore used throughout for mammalian muscle.

Animals were perfused with 10% formalin solution through the left ventricle immediately after killing (usually with chloroform), and the surface of the muscles to be stained was cleared as thoroughly as possible of fascia, avoiding injury to the muscle fibres. The muscles were then immersed in fixative solution until the period of fixation was completed. Again there was found to be considerable latitude in the requirements of the various mammalian muscles; whole mounts presented a good appearance after staining as fresh specimens,

and adequate staining has been achieved after more than twelve hours fixation. One hour was taken as the minimum time for fixation, however, and after washing in water, the preparations were placed in suitably shaped receptacles so that they were completely immersed in the incubating fluid. It is important that the staining fluid has free access to all parts of the surface of the preparation; if part of this surface is in contact with the walls of the receptacle, staining will not take place. A certain minimum quantity of staining fluid is necessary for each specimen, and with large whole mounts, care was needed to ensure that more than this amount was present; otherwise staining was patchy, or of poor intensity.

Staining was at room temperature, for two hours at least and usually overnight. With the longer incubation, some diffusion of the stain at the enzyme sites took place, but this is no disadvantage in whole mounts, increasing the contrast of the endplates against the background muscle which may also be dark after long staining.

After staining and washing, preparations were soaked in 10% formalin solution for several days, the solution being changed whenever it became cloudy. They were then further washed in water, and stored in pure glycerol. Passage through various graded dilutions of glycerol, such as were used for delicate teased specimens and frozen sections, was not necessary with whole mounts. After some months in glycerol, superficial clearing of the specimens had occurred, and this

made photographic reproduction easier and more effective. Xylene was found less satisfactory as a clearing agent for whole mounts.

Material

The animals studied were three small rodents (rat, mouse, hamster), the rabbit, cat, and dog. A small number of muscles from a human foetus were also stained. For purposes of comparison, the diaphragm, and at least one lower limb muscle, were stained in each of the animals used, and a further series of muscles was taken at random from each.

Observations

Rodents :

The distribution of endplates was found to be identical in muscles from the mouse, rat and hamster; no exceptions have been found to this in the muscles studied, and results will therefore be described for the rat, though a preparation of hamster and one mf mouse is illustrated.

The muscles of the rat are characterised by the regularity of the arrangement of their motor endplates. These are invariably grouped in discrete zones running across the muscle belly transverse to the line of the muscle fibres. In most muscles, including the diaphragm, a single band of endplates is present, situated midway between the two attachments of the muscle and running parallel to them. Thus, in a strap muscle such as sterno-hyoid, the visible

band of endplates (fig. 78) runs across the muscle belly at right angles to the main axis of the muscle. In a fan-shaped muscle such as adductor magnus (fig. 79 - A.M.), the endplate band is curved, the curve running parallel to the wider attachment of the muscle. The single band of endplates in the rat diaphragm (see comparable illustration of hamster - fig. 80) curves round the muscle belly midway between the peripheral and central tendinous attachments of the component muscle fibres. A single band is present (fig. 81) in each of the rat jaw muscles, though the band in temporalis is staggered. One band lies between each of the tendinous attachments of the rectus abdominis (see fig. 82 of mouse).

At least three muscles in the rat possess two bands of endplates. These muscles are sterno-hyoid (in which the second band is deep to the sternum - fig. 83), anterior gracilis (fig. 79 - A.G.), and caudo-femoralis. The bands are again transverse to the long axis of the muscle, and are positioned approximately equidistant from each other and from the muscle attachments - they lie, therefore, about the junctions of the muscle thirds.

Only one muscle in the rat has been found to have more than two bands of endplates. This muscle is semitendinosus, where three endplate bands can be shown. The third band is, however, across the secondary head of origin of the muscle, which is separated from the main muscle shaft by a small tendinous intersection.

There appears to be little variation in the endplate distribution in any one rat muscle. From a series of thirty adult rats, the muscles of the neck and of both hind limbs were stained (using the azo-dye method), and only three muscles, as described above, showed two bands of endplates. In muscles having a single band, the endplates were always approximately at the centre of the muscle belly, while in those with two bands, these were evenly spaced from one another and from the muscle ends. The distribution of endplates in the rat is determined before birth, since a one-day old and a three-week old rat have been found to show the same pattern as the adult animal.

In bands of endplates from muscles of adult animals, there is frequently some irregularity of the band. This can be seen in the illustration of part of a hamster diaphragm (fig. 80). This scatter is less marked in the muscles of younger animals.

The arrangement of the component muscle fibres in a single band and a double band muscle were studied. Sterno-mastoid was selected to represent the single band muscles, and anterior gracilis was taken as an example of those with two bands. The number of fibres in transverse sections through the muscle bellies was estimated at a series of points along each muscle. The following procedure was adopted in each case. As soon as the animal was killed, the muscles were

fixed in situ, then carefully dissected free together, where necessary, with a strip of surrounding tissue. They were then washed, dehydrated, cleared in terpineol, and embedded in paraffin. Serial transverse sections were cut at 10/u thickness along the whole length of the muscle belly, and every fiftieth section was mounted and stained with Wilder's reticulin method. These sections were then projected onto large sheets of paper, and the number of muscle fibres in each section counted by marking the fibre with one hand and manipulating an electrical counter simultaneously with the other. The results are shown as graphs (figs. 84 and 85).

The graph of results from sterno-mastoid rises and falls steeply, but elsewhere maintains a steady level. That of anterior gracilis, on the other hand, rises and falls more gradually, and the central plateau is interrupted by a peak; the highest point on this peak is less than double the level of the plateau section on either side. The peak area represents approximately the middle third of the muscle.

To complement the results from anterior gracilis fibre counts, the average fibre diameter was calculated for four sections corresponding to each of the two level areas on the graph, and eight sections corresponding to the central peak. The results are shown in fig. 86. It can be seen that the figures for any one of the three groups are fairly consistent. Those from the two level areas are considerably higher than the results from the central peak area. The average for the

level area nearer the tibial attachment is rather higher than that for the corresponding area near the proximal end of the muscle. It may be pointed out that serial sections through the muscle show a gradual change of outline as they proceed distally, the muscle belly becoming thinner and wider as the tibial attachment is approached.

The results from sterno-mastoid indicate that its component muscle fibres are extending from end to end of the muscle. The results from anterior gracilis indicate that most fibres are ending in the middle third of the muscle. This will be more fully considered when the results are discussed.

Rabbit :

The muscles studied from adult rabbits were

diaphragm
masseter, temporalis
lateral rectus oculi
sterno-mastoid, sterno-hyoid
sartorius
biceps femoris
semitendinosus
semimembranosus
adductor longus
tibialis anterior;

and from a rabbit foetus, the hind limbs and the diaphragm were stained.

In the rabbit diaphragm, a band of endplates is present which lies, as in the rat, midway between the peripheral and central tendinous attachments of the muscle fibres. The band is irregular, however, and in its posterior extent invariably

divides into two bands which separate from one another till they lie apart with perhaps one fifth of the breadth of the muscle between them. This is a regular pattern, confirmed by the study of eight specimens.

A number of muscles in the rabbit possess a single band of endplates. This is the case in the two jaw muscles studied, masseter and temporalis (fig. 87), and a single band is also found in the hind limb muscle, semitendinosus.

In the remainder of the rabbit muscles studied, the endplates were distributed throughout the whole extent of the muscles, in short bands of endplates lying transverse to the long axis of the muscle. This is shown in the illustrations of the rabbit tibialis anterior (fig. 88). To see if the appearances of a paired muscle were mirror images of one another, the right and left tibialis anterior muscles were stained from one animal; these are shown in fig. 88 A. It can be seen that, while the two patterns are not dissimilar, there is no exact mirroring of the endplate patterns. To see if the distribution for any one muscle was constant for different animals, a second pair of tibialis anterior muscles was stained and compared with the first (fig. 88 B). Again there is some resemblance in the endplate arrangements, but no exact duplication. These observations were repeated for the rabbit sartorius, and similar results were obtained, showing that while the scatter of endplates in any one muscle is not entirely random, it is subject to some variation.

As has been observed, the endplates in many rabbit muscles occurred in short broken chains running across the muscle belly. The appearance suggested the possibility that there might originally have been continuous bands of endplates across the muscle, these bands becoming broken up as a result of the muscle fibres altering their positions relative to one another in the course of growth of the muscle. To see if, in fact, the endplates in foetal rabbit muscles were in bands, the hind limbs of a foetal rabbit were (fig. 89) stained, and a scatter apparently asprofuse in its distribution as that in the adult was found. The foetal diaphragm, too, shows a pattern comparable to that found in the adult rabbit.

Cat :

Muscles studied from adult cats and from a six-week old kitten included :

diaphragm
temporalis, masseter
tenuissimus
semitendinosus
semimembranosus
sartorius;

the diaphragm of a foetal kitten was also stained.

A single band of endplates is again present in temporalis and masseter. In all the other muscles studied, including the diaphragm, the endplates are in short scattered chains (figs. 90 - 94), and this scatter appears even more profuse than that encountered in many rabbit muscles. There is no single band at any point in the diaphragm.

Again, the same muscle from different animals shows a similar but not identical endplate distribution.

The diaphragm of a foetal kitten was stained and mounted on a slide in balsam. Staining was weak, but it could be seen that a liberal scatter of endplates was present, with again no indication at any point of a single band.

Dog :

Specimens of the following muscles were obtained from adult dogs :

diaphragm
sterno-mastoid, sterno-hyoid
temporalis, masseter
tibialis anterior.

Staining of these dog muscles produced very unsatisfactory results. The contrast was poor at the endplate sites, and while the endplate distribution could in most cases be ascertained, photographic reproduction was unsatisfactory (see fig. 95). Alteration of the pH of the incubating medium did not improve the staining, and it is probable that the fault lay in using insufficient staining fluid for the bulky specimens. The azo-dye method was tried using more liberal quantities, but results were never good.

It could be seen, however, that many dog muscles have a scattered distribution of their endplates. In the diaphragm of an adult Boxer, endplates were found at all levels along the muscle fibres, and the tibialis anterior illustrated (fig. 95) shows a picture similar to that of the same muscle in the rabbit.

Human Foetus (200 mm. C.R.L.) :

The diaphragm, biceps brachii, and sartorius were stained. The specimens were obtained five hours after death, and were fixed for one hour by perfusion with, followed by immersion in, 10% formalin. Incubation was at pH 5 for twelve hours.

Endplates in biceps are only present at the mid-zone of the muscle, where they form a fairly regular band. In sartorius (fig. 96), the endplates are grouped into clusters, there being five or six such clusters at intervals along each strip of muscle examined; no endplates are seen between these sites in slender strips. Endplates in the diaphragm are predominantly in the region midway between the two attachments of the muscle fibres, but in places the line of endplates is extremely irregular (fig. 97), and many isolated collections of endplates can be seen.

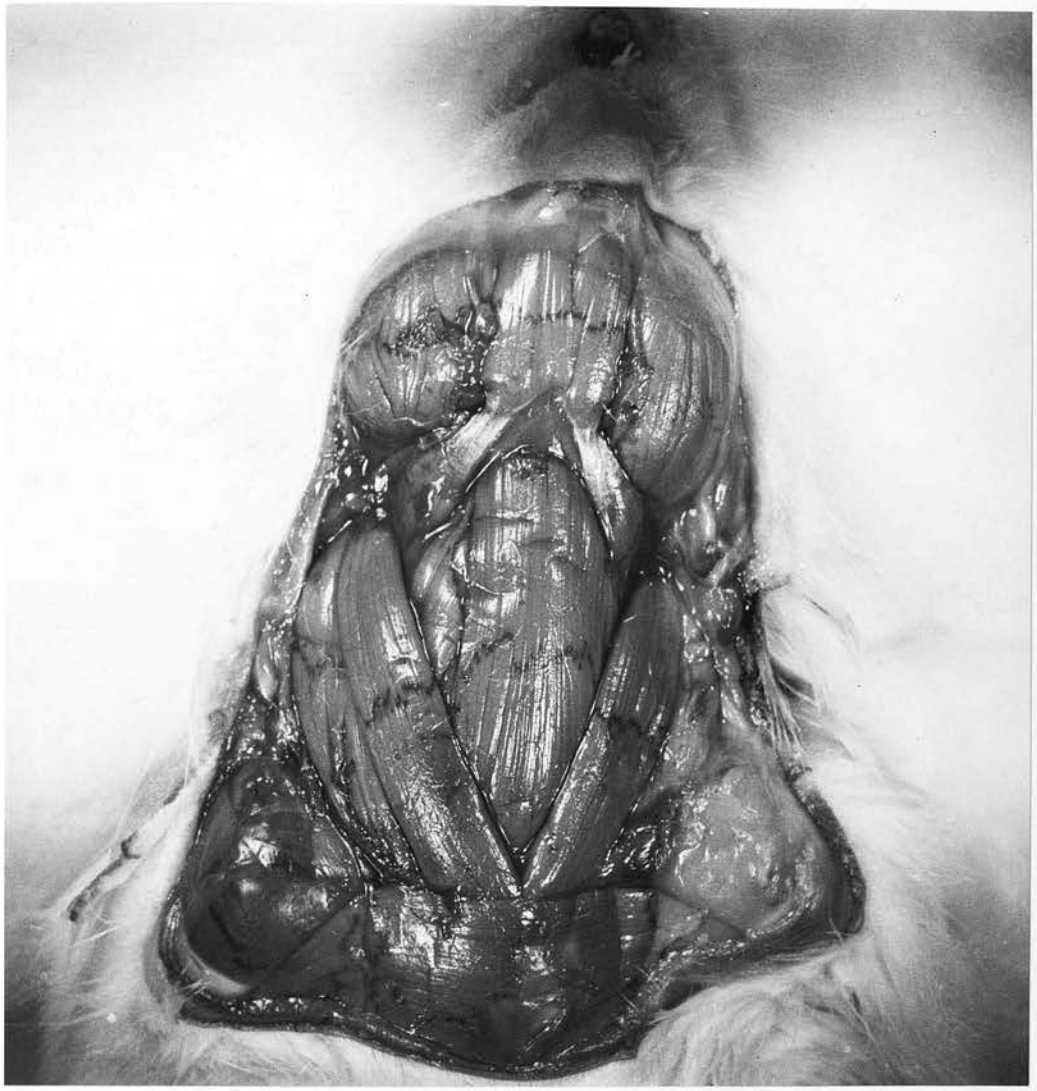


Fig. 78 : Rat (acetylthiocholine) - motor endplates in muscles of neck. (x 3)

Fig. 79 : Rat (acetylthiocholine) - motor endplates in muscles of medial aspects of hind limbs. (x 2)

Fig. 80 : Hamster (acetylthiocholine) - portion of diaphragm. (x 7.5)

Fig. 81 : Rat (acetylthiocholine) - jaw muscles.
(x 3)

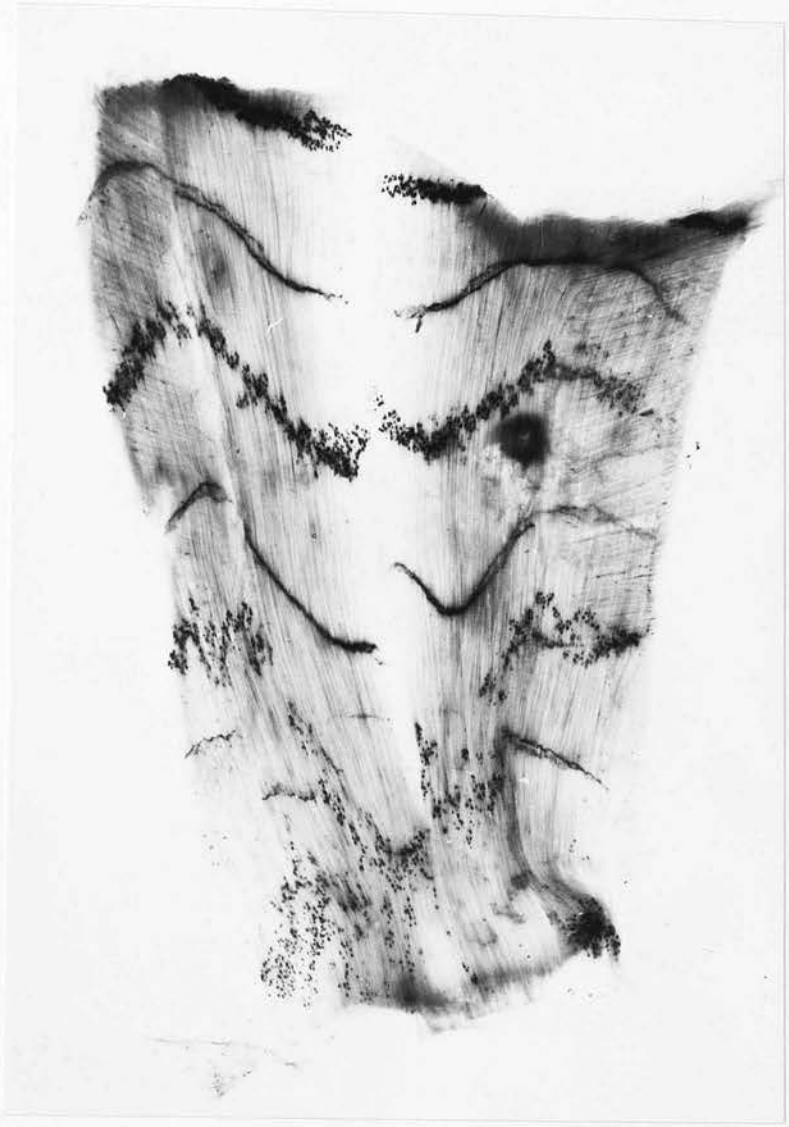
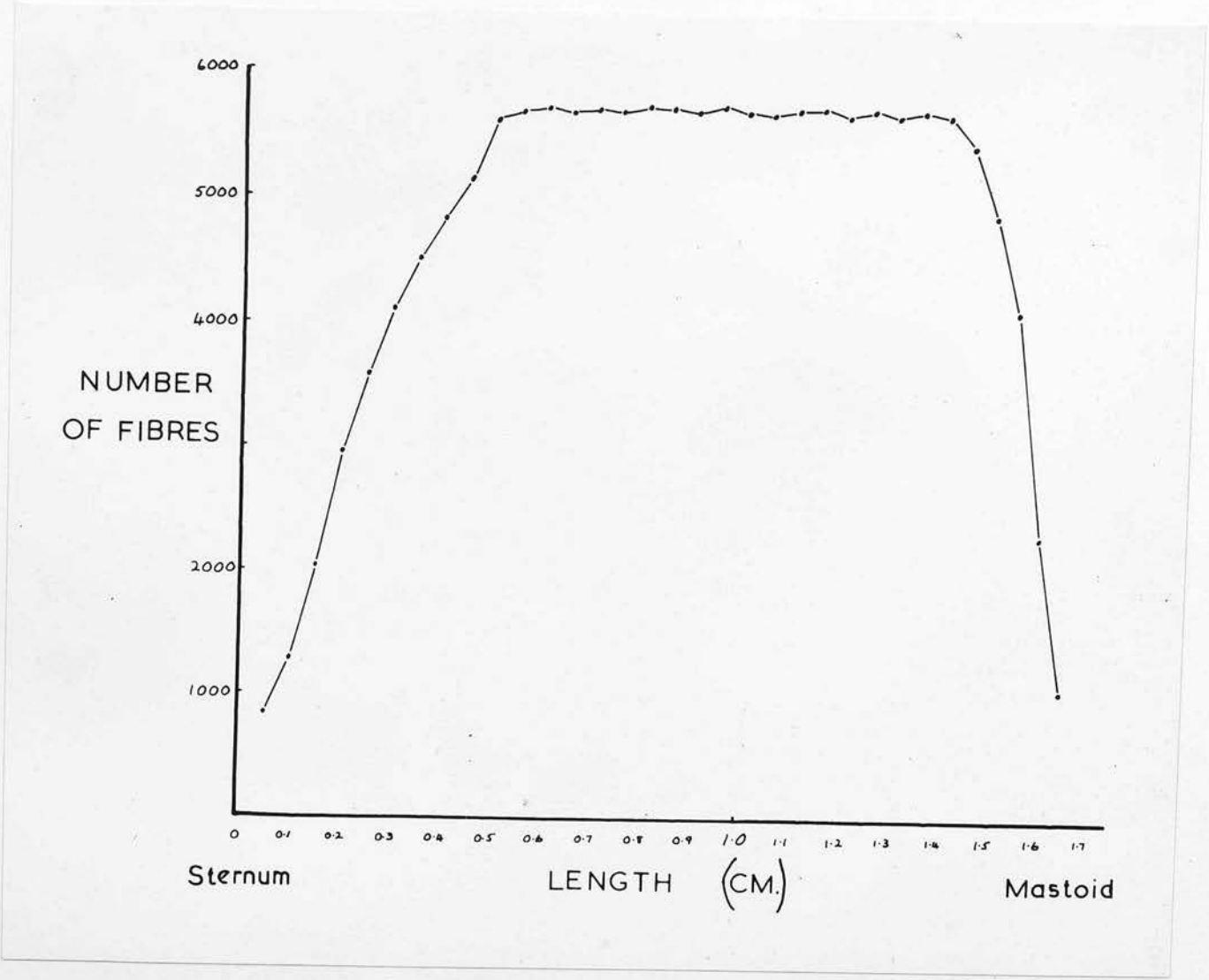


Fig. 82 : Mouse (acetylthiocholine) - rectus abdominis; the tendinous intersections are conspicuous by virtue of the myotendinous staining. (x 6)

Fig. 83 : Rat (acetylthiocholine) - muscles of lower pegion of neck, with a window cut in sternum to show the second band of endplates in sterno-hyoid. (x 7)

Fig. 84 : Rat - serial transverse section fibre counts for sterno-mastoid.



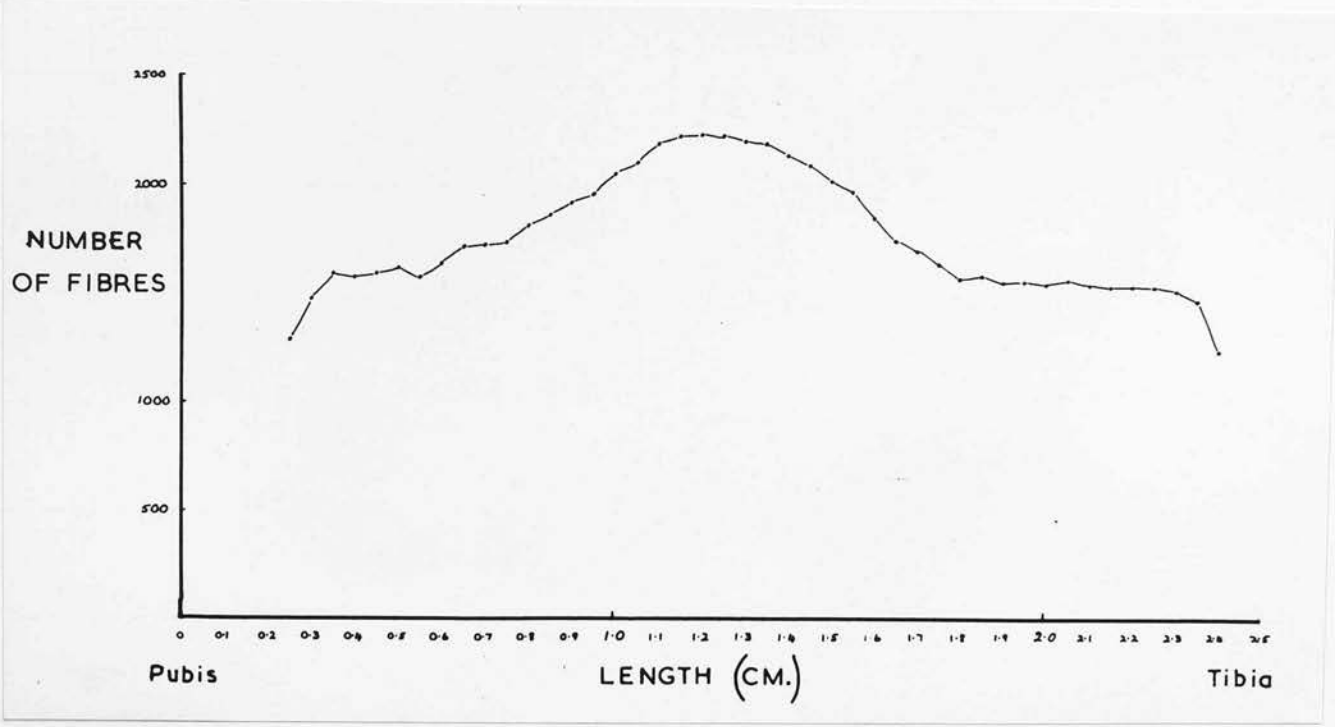


Fig. 85 : Rat - serial transverse section fibre counts for anterior gracilis.

Average Fibre Diameters in Transverse

Sections of Anterior Gracilis.

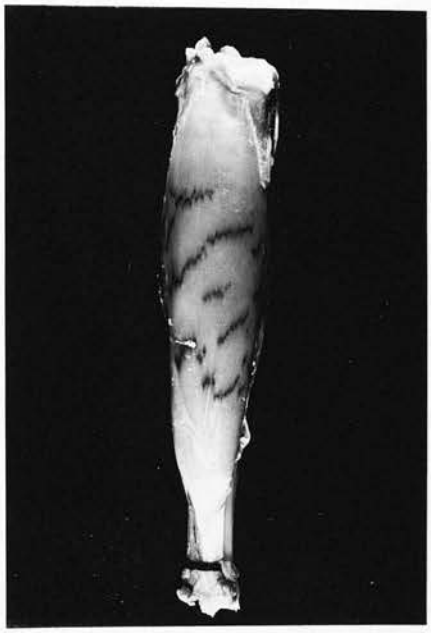
The average fibre diameter was calculated for four sections from the level portions at each end of the graph on Plate 4; and eight sections from the central raised part of the graph.

	Total No. of fibres counted	Total Wt. in mgm.	Average cross-sectional fibre area (sq. μ)
Pubic End	350	1105	52.2
	442	1390	52.1
	453	1460	53.4
	590	1955	54.0
Central Peak Area	762	1680	35.9
	757	1620	34.9
	583	1340	38.0
	738	1630	36.6
	932	2220	38.8
	883	1930	35.1
	804	1905	38.6
	850	1920	36.8
Tibial End	513	1470	47.5
	574	1600	46.2
	406	1150	47.0
	459	1290	46.6

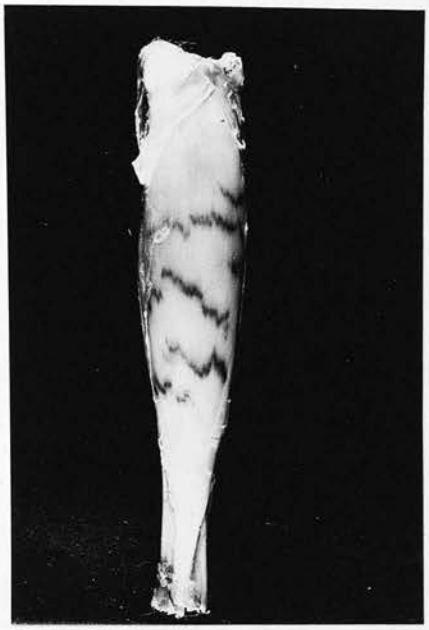
Fig. 86 : Rat - average fibre diameters in transverse sections of anterior gracilis.

Fig. 87 : Rabbit (acetylthiocholine) - masseter and temporalis. (x 2)

Rabbit A

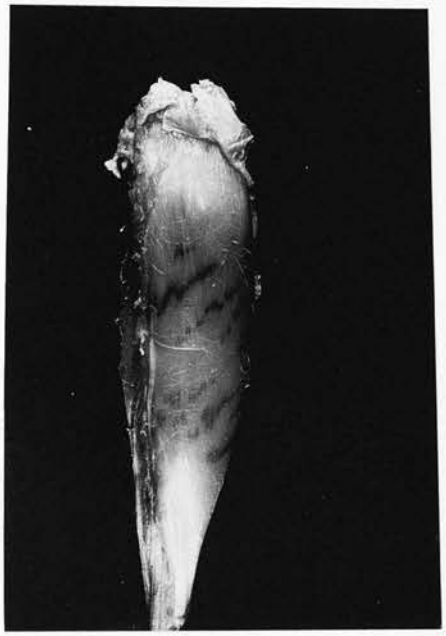


Right



Left

Rabbit B



Right

Left

Fig. 88 : Rabbit (acetylthiocholine) - tibialis anteriors. (x 0.75)

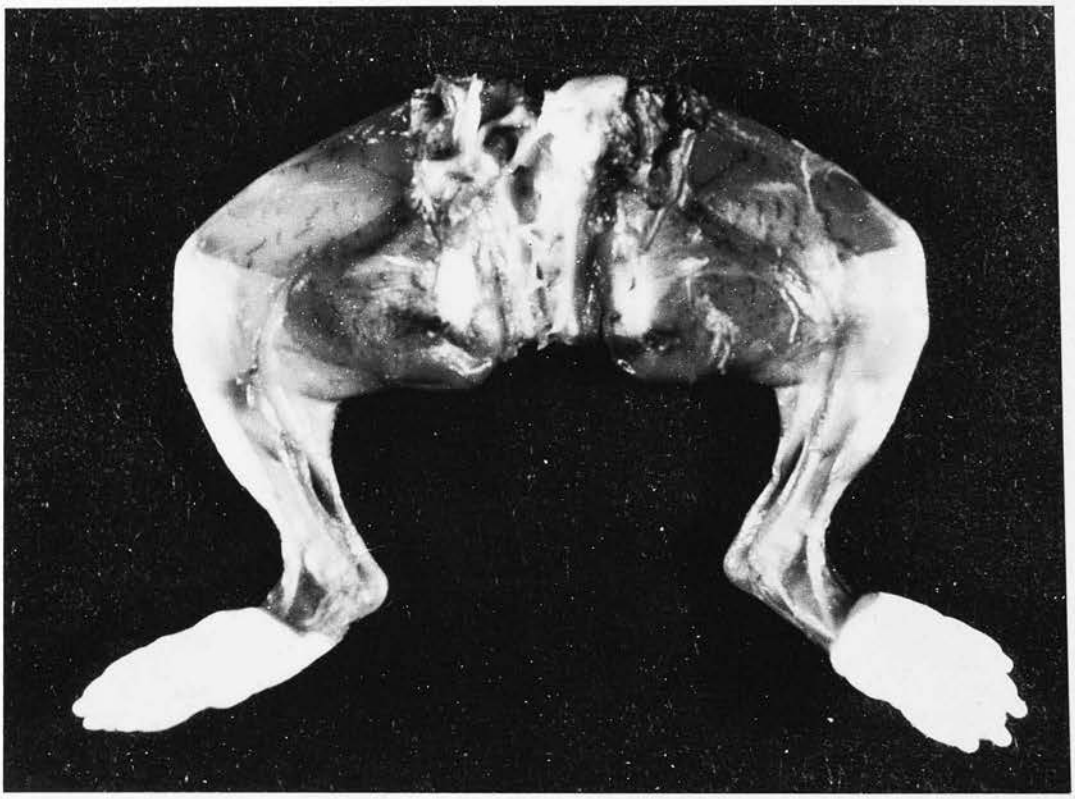


Fig. 89 : Rabbit foetus (acetylthiocholine) - hind limbs. (x 7.5)

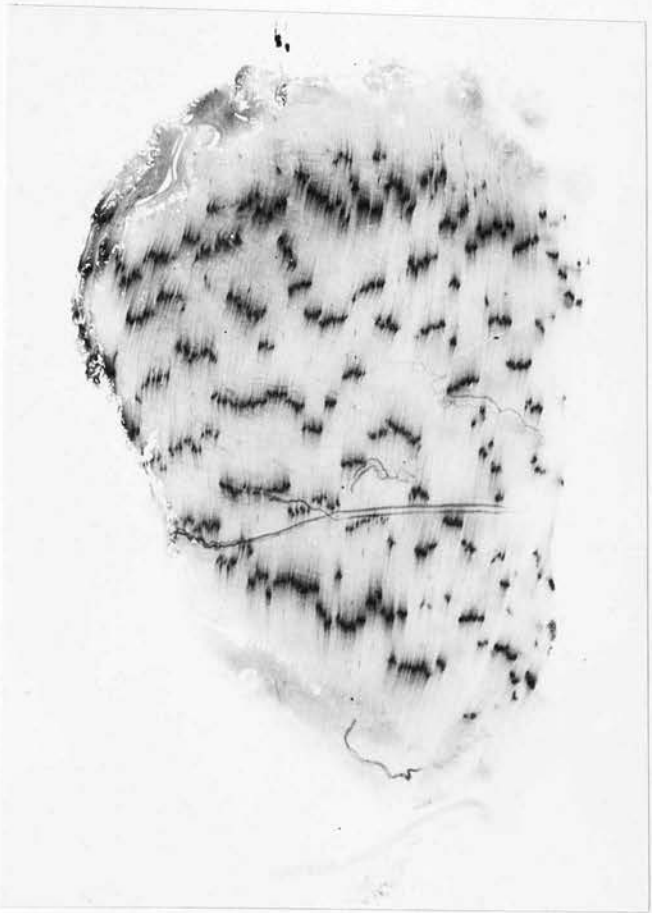


Fig. 90 : Cat (acetylthiocholine) - sartorius. (x 1.5)

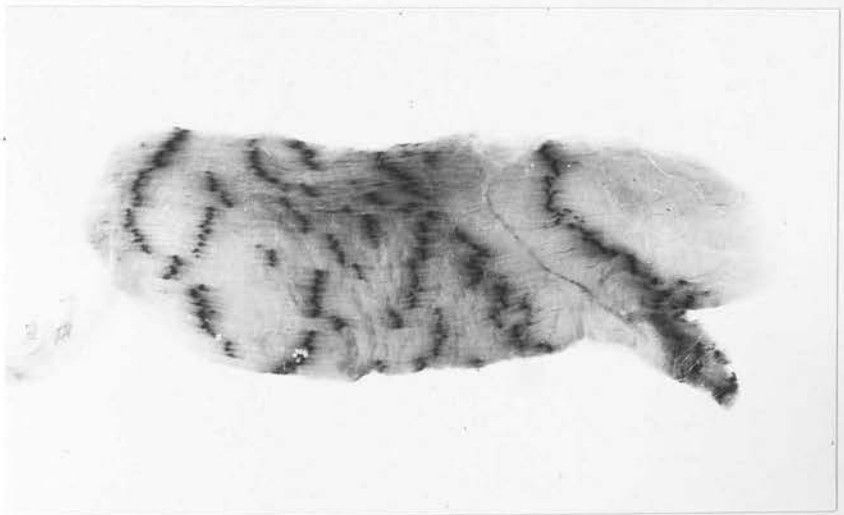


Fig. 91 : Cat (acetylthiocholine) - semitendinosus. (x 1.5)

Fig. 92 : Cat (acetylthiocholine) - tenuissimus. (x 5)

Fig. 93 : Kitten (acetylthiocholine) - medial aspect of fore limb. (x 1.7)

Fig. 94 : Kitten (acetylthiocholine) - diaphragm. (x 1.4)

Fig. 95 : Dog (acetylthiocholine) - tibialis anterior. (x 2)

Fig. 96 : Human foetus (acetylthiocholine) - segment of diaphragm. (x 3)

Fig. 97 : Human foetus (acetylthiocholine) - strip of muscle fibres from sartorius, showing clusters of endplates; this strip is approximately half the length of the muscle; (x 5)

Discussion

1. The Histochemical Demonstration of Cholinesterase.

Primarily from the work of Dale and Feldberg (1934), it is known that acetylcholine acts in the transmission of excitation from motor nerve to striated muscle fibre. The small amount of this substance released at the neuromuscular junction is destroyed, in the brief refractory period which follows the passage of excitation, by the enzyme cholinesterase, which is principally located at the level of the subneural apparatus. Histochemical procedures which demonstrate cholinesterase may therefore be used to display the sites of motor nerve terminations in skeletal muscle.

It is known that more than one variety of cholinesterase is present in tissues, and Augustinsson and Nachmansohn (1949) classify these as true, or acetylcholinesterase, and non-specific, or pseudocholinesterase. The two differ in certain chemical properties such as optimum pH, and may be distinguished by the use of both selective substrates and selective inhibitors. They differ also in their localisations in tissues, and both are present at motor endplates, though according to Koelle (1950), the enzyme at this site is almost exclusively acetylcholinesterase.

The first method introduced for the demonstration of cholinesterase is that of Gomori (1948), wherein the tissue cholinesterase hydrolyses a higher fatty acid ester of

choline. A disadvantage of this procedure was the failure of some acetylcholinesterases to react with the substrate.

Koelle and Friedenwald (1949) employed acetylthiocholine as substrate, this substance being hydrolysed by the tissue cholinesterase even more rapidly than acetylcholine itself. The liberated thiocholine was then made to react with a copper salt to give the relatively insoluble copper thiocholine - a white precipitate which could be observed microscopically, or could be converted to black copper sulphide by the addition of ammonium sulphide. A disadvantage of this method is the considerable amount of diffusion which occurs at the reaction sites, presumably of the products of hydrolysis rather than the enzyme itself since this has a molecular weight of over 1 million. A further shortcoming is the need to use fresh frozen sections, since these may undergo considerable histological alteration in the course of the staining process. Various modifications have been introduced to try to overcome these disadvantages, notably control of the pH of the incubating solution and of the duration of incubation, and the method may now be used on carefully fixed tissues to produce fairly accurate localisation of the enzyme. A much modified version of the technique has been produced by Holmstedt (1957), in which the final step of conversion of copper thiocholine to the black copper sulphide is omitted; this gives more accurate localisation, since in this step the copper thiocholine is dissolved by the

ammonium sulphide and the copper sulphide may not appear in precisely the same situation.

The second method used in this work to demonstrate cholinesterase at motor endplates is the modification, by Lewis (1958), of a technique first introduced by Menten, Junge, and Green (1944) and subsequently extended by Nachlas and Seligman (1949). Tissue is incubated with a naphthyl acetate, and from the hydrolysis of this substrate by cholinesterase, naphthol is liberated; this is then acted upon by a suitable diazonium salt, to produce a brightly coloured precipitate of an azo-dye at or close to the site of enzyme activity. The original method employed beta naphthol acetate, and produced much diffusion, but this was minimised by the substitution of alpha naphthyl acetate which Denz (1953) shows to give good results on motor endplates. An advantage of this method over the acetyl-thiocholine procedure is the good penetration of the incubating solution into most tissues, and Lewis (1958) has made the technique applicable to relatively thick sections and small whole mounts which, with the first method, would stain only in their superficial layers.

Penetration of the incubating medium in the azo-dye technique is greatly limited by lipid membranes, and the ability of indoxyls to pass through these membranes is an asset of the most recently introduced histochemical procedure for cholinesterase (Barrnett and Seligman, 1951; Holt, 1952).

Illustrations of results obtained with this method indicate that it gives very clear and accurate localisation of cholinesterase in motor endplates, but it was not possible to obtain the necessary materials to allow its use in the present work.

It was observed earlier in this work that the results of staining with the acetylthiocholine technique depend to a great extent on suitable staining conditions. Modification of a number of factors may considerably alter the quality and degree of staining, and some aspects of these will now be discussed.

Fixation of tissue prior to histochemical staining is desirable if satisfactory histological preparations are to be obtained, and most of the commonly used fixatives rapidly inactivate cholinesterase. In tests to find a fixation process which would leave sufficient residual activity in the tissue to allow its detection histochemically, formaldehyde has been found the most suitable (Couteaux and Taxi, 1951, 1952; Holt and Withers, 1952). The work of Ravin, Tsou and Seligman (1951), and of Taxi (1952) indicates that the enzyme resistance to formaldehyde varies according to the type of tissue used; thus in mammalian motor endplates, a fairly lengthy fixation may still permit the detection of cholinesterase activity. In the present work it was found that even after twelve hours immersion in 10% formalin, motor endplates could be recognized in rat muscle; and Coers (1953)

has obtained good results in human muscle after eight hours fixation. It was found in the work on avian muscle, that muscle fibres from the chick posterior latissimus dorsi ('en plaque' endings) were better able to withstand fixation than those from the anterior latissimus dorsi ('en grappe' endings), and Couteaux (1954) points out that inactivation of cholinesterase by formaldehyde is extremely rapid in frog muscles and more so in the 'en grappe' endings of the slow fibres than in the 'en plaque' terminations on twitch fibres.

Lewis (personal communication) has adopted a technique of fixation whereby enzyme inactivation is reduced, in which after immersion in formalin solution at room temperature for a short length of time, fixation is continued up to one hour at 4° C. in the refrigerator.

Incubating solutions in the present work have been maintained at room temperature, since this was found to produce less diffusion of the stain at sites of enzyme activity. Couteaux (1955) points out that this has the further advantage of slowing the histochemical reaction, so that the optimum incubation time may more easily be assessed.

The type of buffer employed is of some moment in determining the degree of diffusion. Lewis (1958) employs tris (hydroxymethyl) amino methane in preference to the phosphate buffer normally used in simultaneous coupling azo-dye techniques. Koelle (1949) employed a phosphate buffer, but considered that this was in part responsible for

diffusion, and preferred subsequently a maleate or acetate buffer. Couteaux (1955) admits that it is difficult to explain the difference in action of the various buffers, but feels that artifacts are not in all cases due to unequal buffer capacity. In avian muscle, it was found that alteration of buffer did not appear to influence the degree of diffusion, but the most satisfactory results from mammalian tissues have been obtained with an acetate buffer.

The duration of incubation varies considerably for different tissues, and must be assessed separately for each and in relation to the other factors influencing the staining process. It has been noticed, however, that most tissues will stain adequately in under two hours provided the incubating solution is well buffered at the optimum pH for the particular tissue. Alterations in pH necessitate much longer periods of incubation, with resultant impairment in the degree of enzyme localisation obtained.

The final factor to be discussed is the pH of the incubating medium. It was noted that of the two parts of the chick latissimus dorsi, the posterior muscle stained at all pH values from 4.5 to 9.5, while the anterior stained only between pH 7.5 and pH 8.5. This difference is presumably due to the different types of endplate on the fibres of the two muscles. In the mouse diaphragm, staining took place at pH 4 to pH 7, and was not investigated outside this range. On the other hand, in the lizard tail, only sections incubated

at pH 5 to pH 7 stained, and in the dogfish the requirements appeared even more critical, endplates being seen at pH 6, and with difficulty at pH 7. The optimum pH for cholinesterase is about pH 8, but Koelle (1950) showed that no precipitate of copper thiocholine is obtained if the incubating medium is buffered at this pH value. Lewis (personal communication) now uses a pH of 5.2 for Mammalian muscle, and a pH of 5 was found in the present work to give good results with most mammals.

Since the optimum pH of cholinesterase is about pH 8, it follows that the more acid the pH, the longer the staining time required for any one tissue. True cholinesterase tends to be inactivated at pH 4.5, and staining of pseudocholinesterase only is obtained as the pH drops further to about pH 2. The optimum pH for the staining of cholinesterase at motor endplates varies for the particular tissue, and is also dependant on other factors influencing the staining procedure. If the pH of the incubating medium is outside the optimum value, other structures tend to stain more intensely. Thus Schwarzacher (1957) points out that while the motor endplates in the mouse and rat are best demonstrated at pH 5, the muscle-tendon junction cholinesterase is seen best if the pH of the incubating medium is raised to pH 6, and Couteaux (1955) observes that alteration of pH from the optimum value increases the staining of nuclear and other structures in the vicinity of the endplate, as well as a small amount in

the terminal portion of the motor nerve; this latter staining is, according to Couteaux and Taxi (1952), the result of a diffusion of the reaction products starting from the subneural apparatus with secondary precipitation at the level of the nerve arborization. Couteaux (1955) states that these effects are only found in fixed tissue.

2. Consideration of Results.

a. Segmental Muscle of Lower Vertebrates.

Amphioxus

The relation of neuromuscular junctions to the muscle plates in Amphioxus does not appear to be decided. Rohde (1888) and Retzius (1891) admitted failure to find any, but Van Wijhe (1893) described triangular-shaped, terminal expansions to the motor nerves running between the muscle plates. A fuller account of these endings was given by Heymanns and Van der Stricht (1898), who described them as being spade-shaped, flat, or cone-shaped. Dogiel (1903) mentioned corresponding 'end-cones' in the same animal. Boeke (1908) commends the Bielschowsky technique for the demonstration of endplates in Amphioxus, and he describes both motor and sensory nerve terminations; the latter he thought to be the terminations of certain fine nerve fibres in ventral roots described by Dogiel as being possibly sensory. Ayers (1921) illustrates motor nerve terminals on muscle plates, where the nerve breaks up on the surface of the plate into a number of terminal fibrils. Bone (pers. commun.) writes that "onto each muscle plate, and in the middle of each, at their edges, lie stuck the spade-shaped end-formations".

Cholinesterase appeared as a series of diffuse streaks within the myotomes and parallel to the muscle plates. It was first thought that this appearance was produced by the staining of nerve bundles, but in silver-stained sections

cut in a similar plane to the frozen sections stained for cholinesterase (i.e. parasagittal), it is seen that only one such nerve bundle appears in any one myotome. On the other hand, the terminal expansions of the motor nerves are scattered fairly evenly throughout the myotomes, often clustered in groups at various positions in the myotomes, but not, as Bone suggests, always at the middle of a muscle plate. It is more likely, therefore, that the diffuse streaks of cholinesterase arise from the accumulated staining of many of these motor nerve terminals, though the areas of stain which merge with myosepta must be produced by staining of nerve bundles entering the myotomes at such points. It is possible that the diffuse character of the zones of stain may be a consequence of absence of a subneural apparatus, such as appears to localise the cholinesterase in the motor endplates of higher vertebrates; no well developed subneural apparatus was found in the lamprey, in fish, or in Xenopus larvae. In the absence of observations on the fine structure of Amphioxus neuromuscular junctions, however, this must remain a hypothesis.

Lamprey

The architectural arrangement of the myotomal muscle of the lamprey has been described on a number of previous occasions. The first appears to be in 1867, when Grenacher produced an account of the subdivision of the myotomes into a series of muscle units, each containing two types of muscle

fibre, positioned as has been described in the present work, but with the central plates represented as syncytia of branching fibres, presumably on account of their fragmentation during histological preparation. Maurer (1894) described the muscle architecture in detail, and his impressive illustrations agree in all respects with the findings presented here. His use of the term 'parietal' to describe the fibres of the ventral and dorsal layers of a unit has been followed. He, too, shows broken central plates in illustrations of transverse sections, but from his text it is obvious that he was in no doubt that they were actual sheets of muscle. Schiefferdecker (1911) appears to base his account on Maurer's description, and reproduces Maurer's diagrams. Tretjakoff (1927) deals with the distribution of ventral roots in the Ammocoete, and illustrates from his methylene blue preparations, the plexus of nerves that lies between adjacent muscle units. Gerebtzoff (1959) has observed the distribution of cholinesterase in the lamprey, and states that it is present "at the proximal end of each broad band of a muscle casket, and in the equatorial region of each narrow band".

The reason for the architectural arrangement of the two types of muscle in the lamprey is obscure. Some information is available in the literature on other cyclostomes, principally Myxine, and two varieties of fibre are again described. The fullest accounts are those of Muller

(1835-45), Maurer (1894), and Cole (1907). All agree that fibres corresponding to the parietal fibres of the lamprey are confined to the ventral surface of each muscle unit, though Maurer illustrates an occasional stray parietal-like fibre on the opposite side of a unit. The second form of muscle in Myxine, corresponding to the central plates of the lamprey, appears to be organised in fibres rather than sheets. The arrangement of muscle tissue in sheets is therefore only found in Amphioxus and the lamprey.

The existence of these two types of muscle may have some functional significance, and a clue to the nature of this may lie in the differences in their respective morphology and innervation. The parietal fibres are seen with the light microscope to have a looser texture than the central plates, and to possess many nuclei, and a richer innervation. The central plates, on the other hand, have relatively little sarcoplasm, few nuclei, and are innervated only at their myoseptal margins. Electron microscopy confirms that the parietal fibres are, in comparison with the central plates, rich in sarcoplasm and mitochondria.

It will be shown, in a later section of this discussion, that a characteristic histological feature of muscle fibres in higher vertebrates which have a tonic or postural function, is this relative richness in sarcoplasm and mitochondria, and it is therefore feasible to postulate that the parietal fibres of the lamprey may have a tonic function, while the

central plates have the responsibility of producing myotomal contractions to effect the rapid lateral-flexion movements of the body in swimming.

Electrophysiological studies on the two types of muscle fibre in Myxine have shown (Anderson and Jansen - personal communication) that the parietal-like and central plate-like fibres respectively behave as tonic and twitch muscle fibres, and it would be interesting to have results of similar studies on the lamprey.

Fish

Literature on the innervation of fish muscle is scanty, and for the most part fairly old. One gathers from it that a wide variety of nerve endings may be found. Amongst the older workers, Stannius (1850) and Fischer (1881) both failed to observe any terminations to the motor nerves, but Krause (1869) described Teleost endplates and stressed the similarity of their appearance to endings he found in Amphibia. According to Krause, Teleost endplates are very long, slender structures, whose long axis lies always parallel to the long axis of the muscle fibre, ending in small button-like swellings. Maurer (1915) also stresses the similarity of Teleost endplates to those of Amphibia. The two main forms of endplate described in fish muscle are a grape-like form and a plate-like variety. Retzius (1892) and Perez (1934) illustrate both types, but Stefanelli (1932) mentions only the grape-like form, and Cole (1955) only finds the

plate-like type. Other workers describe less typical motor nerve endings. Arndt (1873) speaks of terminal networks of fine nerves round the muscle fibres, Cavalie (1902) describes 'pseudo-plates', Okamura (1929) states that ganglion cells only are to be seen in the goldfish and carp, and Kirsche (1948) mentions coarse medullated fibres forming motor endplates with extremely delicate fibres forming 'accessory endings' within the endplates. Basket endings in fish have been described by Giacomini (1898), Perez (1934), Couteaux (1950), and Barets (1952).

Few of these workers mention the positions of the endings on muscle fibres, apart of course from those who describe basket endings; their main object is description of the morphology of the endplate. From the present work, it can be seen that the site of the endplate may vary considerably. Thus in the dogfish, most myotomal muscle fibres show paraterminal zones of cholinesterase which correspond to the sites at which motor endplates were found with the electron microscope. In the six species of Teleost studied, however, the enzyme was found at all levels in the myotome, and nerves (silver-staining) and endings (electron microscopy) were seen to be as freely distributed. While there appeared in these species to be only one form of innervation in the myotomal muscle, this is not always the case. Barets (1952), in the catfish, describes three separate varieties of innervation - terminal, central, and scattered - two of these

forms at least were present in the deep lateral muscle. Gerebtzoff (1959) has observed two forms of innervation in Teleosts, using the acetylthiocholine technique for cholinesterase.

In this work, a different type of innervation was present in the superficial muscle of the dogfish from that in the deep lateral muscle, and the finding that only one form of innervation was present in the Teleosts may be on account of their being young animals whereas the dogfish was almost fully grown. The observation that the parietal fibres of the muscle units in the lamprey are rudimentary in the Ammocoete, may be relevant in this connection, since it may be that these narrow fibres in the lateral superficial muscle of fish develop only in the older animal. It may be, however, that such fibres are never well developed in these Teleosts.

Amphibia

The myotomes of all five species of Amphibia display an identical pattern of cholinesterase distribution. The enzyme is present only at the ends of the muscle fibres, and each muscle fibre is innervated at both ends. Nerves from the myoseptal plexuses run to the ends of the muscle fibres and envelop their tips.

This type of neuromuscular junction is described by the Italian, Giacomini (1898), who calls such endings "terminazione nervosa a paniere". Retzius had, six years earlier, observed terminal innervation in the cyclostome, Myxine glutinosa.

They were described by Giacomini in Selachians as well as Amphibia, and Ceccherelli (1904) found similar endings in the adult Anuran, Bombinator igneus. More recently, Lewis (1957, 1958) has demonstrated with his azo-dye method that cholinesterase activity is confined to the ends of the myotomal muscle fibres in Xenopus larvae.

Giacomini (1898) attributes a sensory function to basket endings, viewing them as analogues of the more highly differentiated muscle and tendon spindles of the Amniotes, and he correlates a lack of basket endings in the Amniotes with the appearance of such spindles. He states that muscle fibres possessing basket endings have, in addition, motor nerve terminals near their mid-points, but these were not observed in this work, and neither Couteaux (1950), nor Barets (1952), finds such terminations. There can be little doubt that in Amphibia at least, the basket endings are the true motor endings of the muscle fibres, since they are the only sites on the fibres at which cholinesterase activity is found, and since the electron microscope has revealed that the nerve endings have a similar structure to those found in the motor endplates of other species and contain the vesicles characteristic of such endings (Robertson - 1957).

It has been found in the course of this work that terminal innervation is present in other Classes of vertebrates. Thus, the central muscle plates of the lamprey have neuromuscular junctions only at their extremities, and it is known from the work of Couteaux (1950) and Barets (1952) that similarily

situated endings are found in certain species of fish. Barets (1952) observed them in three out of twenty-four Teleosts studied. The basket endings in Amphibia may be contrasted with those of the lamprey and of fish, in some respects. Thus, the nerve endings on the lamprey central plates are found, with the electron microscope, to be positioned close to, but not on, the line of union between muscle and myoseptum, whereas in Xenopus, the endings may lie between the sides of the sarcolemmal clefts at the muscle-tendon junction or applied to the sarcolemma between the clefts. No discrete localisation of the cholinesterase activity has been observed in Amphibia, the zone of dye at the end of a fibre always being diffuse; as was suggested in connection with Amphioxus, this diffuse appearance may be a consequence of the poorly developed subneural apparatus found in lower vertebrates. While terminal innervation was only found in some of the muscle fibres of a small proportion of the series of Teleosts studied by Barets (1952), it is the exclusive form of innervation of the muscle fibres of the five species of Amphibia studied in this work.

It is probable that some of the cholinesterase staining at the ends of the segmental muscle fibres in Amphibia is the result of a cholinesterase response by the muscle-tendon junction, since there is no morphological difference between this site in Amphibia and in other species. At the same time, a proportion of the staining may be attributed to the

neuromuscular junctions, which are so intimately related to the line of union of muscle fibre and myoseptum in Amphibia.

It has been pointed out that fibres having basket endings are doubly innervated, and the electrophysiological studies of Cuypers and Fessard (1952) suggest that this may be a consequence of the intrinsic properties of the muscle fibres. They show that when the nerve at one end of a doubly innervated fibre (in the catfish) is stimulated, the resulting action potential rapidly decreases in intensity as it progresses along the fibre, and they conclude from this that these muscle fibres are only capable of a local response to nerve stimulation, and are unable to propagate an impulse for more than a very short distance.

Reptiles

It has been observed that the motor endplates in snake segmental muscle lie at or near the middle of the muscle fibres, whereas in the tail of the lizard they may be found at any point along the fibres and frequently are close to the muscle-tendon junction. It has also been noted that the cholinesterase pattern in lizard muscle bears a strong resemblance to that shown by Couteaux (1958) and others for mammalian endplates, whereas the zone of cholinesterase at the site of a snake endplate shows no structural detail and has a regular outline.

The literature appears to contain relatively few observations on the innervation of Reptilian musculature. As regards the snake, Tiegs (1953) states that he has observed 'en grappe'

endings in the segmental muscle of the python. Both 'en grappe' and 'en plaque' endings were found by Boeke (1931 - python), Kulchitsky (1924 - python), Hines (1932)- boa constrictor), and Cole (1955 - Coluber). None of these writers makes reference to the position of the neuromuscular junctions on the muscle fibres.

Negro (1890) studied Saurian muscle, and found the two forms of ending present. He states that two motor endplates were often present on the same muscle fibre, but states that these were rarely both the 'en grappe' form of termination. Other workers who have described both types of endplate in lizard muscle include Rossi (1902), Rouget (1902), and Cole (1955).

The observations of the workers cited above were not confined to the segmental musculature of the species studied, and therefore do not conflict with the findings in the present work, where only one form of ending was observed in the snake, and similarily in the lizard. The cholinesterase pattern in the lizard neuromuscular junctions is identical to that of mammalian 'en-plaque' terminations as illustrated by Couteaux (1958). The consistently oval and regular zone of cholinesterase at endplate sites in the snake suggests that these differ in their morphology from the lizard endings, but further study is necessary to confirm this.

The scatter of endplates in the lizard tail suggests that the muscle-nerve relationship may represent a transition between the types found in the lower and higher forms of vertebrates, for in the higher vertebrates, as in the intercostal muscle of the snake, the endplates are situated somewhere near the middle of the muscle fibres. The observations of Hughes and New (1959) on the regeneration of the tail of the lizard Sphaerodactylus are important in this connection. They found that although the original tail muscle fibres have their endplates towards their middle, in a regenerating tail the muscle fibres are at first only innervated at their ends, at which time they give a cholinesterase reaction similar to that of the myotomes of Urodeles and larval Anura. Later, however, the nerve fibres grow into the myotome to give rise to the pattern present in the original tail (Lewis and Hughes, 1960).

The scatter of endplates in the lizard tail, while showing an anatomical transition between lower and higher forms, may indicate some degree of functional inefficiency when compared with mammalian muscle fibres where the endplates are near their midpoints. It would be interesting to know if corresponding differences in physiological behaviour are present.

b. Avian Muscle.

It has been demonstrated histochemically that two different types of innervation are encountered in the skeletal muscle fibres of the chick. Of the nine muscles examined, five showed the presence of both types of muscle fibre; three contained only focally-innervated fibres; and one muscle, the anterior latissimus dorsi, yielded only fibres with many neuromuscular junctions (for convenience referred to as 'multiply-innervated' fibres).

Segments of many hundreds of muscle fibres from the posterior latissimus dorsi of the chick were examined, the longest obtained being 1.7 cm., and in no case has more than one endplate been found on a single fibre. Nor was more than one endplate found on the focally-innervated fibres from other muscles investigated. This does not exclude the possibility of more than one endplate existing on a focally-innervated fibre, but it can be said that if more than one does occur, the interval between the endplates is much greater, by a factor of ten at least, than the intervals between neuromuscular junctions on multiply-innervated fibres.

The considerable variation in individual values for the distance between adjacent endplates on fibres from the anterior latissimus dorsi is illustrated in fig. 77, and it has been pointed out that this may in part be due to some endings being lost in the course of isolating fibres. There does, none the less, appear to be a range of intervals between

endplates on the same fibre. When the average interval is calculated, and graphed against the total length of the muscle, for different sizes of bird, the points fall on a straight line which passes through the origin of the graph (fig. 76). From this it may be concluded that the number of endings on a chick multiply-innervated fibre is relatively constant, and that this number bears a constant relationship to the length of the muscle as a whole. It is known (Ginsborg, 1960) that at least some of the muscle fibres in the anterior latissimus dorsi extend the length of the muscle, and it may be calculated that the total number of neuromuscular junctions on these fibres is about eighty.

A corollary of these findings is that the neuromuscular junctions on a multiply-innervated muscle fibre separate from one another as the muscle increases in size, the rate of separation being proportional to the rate of growth of the muscle. This will be referred to further in the section on the growth of muscle fibres.

The distribution of focally- and multiply-innervated fibres amongst the various muscles examined supports the idea that the peculiar type of physiological response known as a 'contracture', such as was first described by Langley (1905/6), and which is induced by acetylcholine-like substances, occurs only in the multiply-innervated fibres. Contractures have been shown to occur in a number of the muscles studied (see for example : Rückert, 1930/1 -

latissimus dorsi of pigeon; Child and Zaimis, 1960 - biventer cervicis, semispinalis cervicis; Ginsborg, 1960 - anterior latissimus dorsi), and multiply-innervated fibres have been found in these muscles. They do not, however, appear to be present in the three muscles examined which do not show contractures (posterior latissimus dorsi - Ginsborg, 1960; extensor metacarpi radialis profundus, extensor digiti III and IV - Ginsborg, 1961).

It does not appear to be known whether contractures occur in bird muscles in vivo, but it would seem to be a possibility that this peculiar type of sustained contraction has some functional value, and that multiple innervation is necessary for the production of this special physiological response.

c. Mammalian Muscle.

It has been shown that most muscles in small rodents possess a single band of motor endplates lying transversely across the middle of the muscle belly. The possible arrangements within such a muscle of the component muscle fibres are as follows. All the muscle fibres may run from one attachment of the muscle to the other attachment, every fibre therefore having an endplate about its midpoint. Or a certain number of fibres may be ending within the muscle though arising from one of the muscle tendons; the endplate will therefore lie some distance from the centre of the fibre. Or some fibres might both begin and end some distance from the muscle attachments, in which case the endplate may still be near the fibre middle.

To investigate this question, serial transverse sections from the rat sterno-mastoid, which has a single band of endplates, were counted. The graph of the results shows a sharp rise and fall in the immediate neighbourhood of the muscle tendons, and in the rest of its length maintains a steady level which indicates that the number of fibres remains constant for most of the muscle length and therefore that the muscle fibres are extending throughmost of the muscle.

That every fibre in the mouse diaphragm has one motor endplate is indicated from some unpublished work by Peters on this muscle. Small strips of fibres were isolated by teasing, and these bundles were stained with the acetylthiocholine technique. They were then trisected, and the two

outer portions embedded in paraffin, sectioned, and stained with Wilder's reticulin method. The muscle fibres in these sections were then counted and compared with the number of endplates counted in the central thirds. Results tallied remarkably, indicating that the band of endplates contained one endplate for each muscle fibre.

The arrangement of muscle fibres in a muscle having two bands of motor endplates was studied, using the rat anterior gracilis, again by counting muscle fibres in serial transverse sections. The resulting graph differs from that of sterno-mastoid in that the central plateau part is interrupted by a further rise to form a peak extending over the middle third of the muscle, though the number of muscle fibres is relatively constant in the outer thirds of the muscle until the muscle attachments are approached. This graph can be explained by supposing that the muscle fibres run from one or other end of the muscle and terminate within the middle third, and such an hypothesis is supported by the results of calculations to determine the average fibre diameter at three points along the muscle belly; these show that the diameter falls considerably in the middle third of the muscle, as would be expected if many fibres were tapering to termination.

Schwarzacher (1947) has studied the distribution of cholinesterase in muscles of the rat and mouse, and the present findings of endplate arrangements correspond closely with his observations. He adds biceps femoris to the three

muscles with two bands of endplates already described, and investigates the arrangement of muscle fibres within anterior gracilis by isolating individual fibres by teasing. His results, from the isolation of over four hundred fibres, indicate that most (89%) of the fibres in this muscle do in fact terminate in the middle third. He states that the remaining 11% extend through the whole length of the muscle and that each of these fibres has two motor endplates. These figures would fit with the fact that the peak of the graph of fibre counts is less than double the level at the plateau segment on either side of the peak, though this could also be explained by a small proportion of fibres not overlapping.

Two questions arising from these results are (1) the significance of four muscles having two bands of endplates as against the single band of the other muscles, and (2) the importance of two endplates on some muscle fibres within these muscles. A possible answer to the first question may lie in the fact that these four muscles appear to be the longest muscles in the rat body, but no functional distinction is known which would separate these muscles from the remainder. The fact that fibres having two endplates are considerably longer than those with single endplates, may be of significance with regard to the second question.

In a few muscles of the rabbit and cat, single bands of endplates have been observed. Thus the masticatory muscles, temporalis and masseter, in both animals, possess a single band. It will be seen from the illustration of these muscles in the rabbit that their muscle fibres are relatively short, and all the muscles studied in both the rabbit and the cat, which have more extensive muscle bellies, show a scattered endplate distribution.

This scatter of endplates might imply a muscle built up of a series of interdigitating muscle fibres, each with a motor endplate at its midpoint. The same picture could be given, however, by fibres extending right through the muscle, each having several motor endplates. A combination of these two is a further possibility.

There are several instances of the arrangement of muscle fibres within such muscles having been determined by isolation of the fibres, after maceration, by teasing. Thus Van Harreveld (1947) studied the sartorius of the rabbit, and reported that no fibre obtained was as long as the muscle itself. He claims that every fibre was isolated from seven muscles, and that the average number of fibres for these seven muscles was 6282, though in cross sections through the muscle the average number of fibres present was only 6% of the average fibre total. As a result of these figures and of his teasing experiments, he states that the muscle is built up of two kinds of fibre, situated serially, fibres attached to

tendon and ending in the muscle substance, and fibres both beginning and ending within the muscle belly. Similar findings were reported by Huber (1916) for a rabbit thigh muscle.

The tenuissimus muscle in the cat (fig. 92), whose fibre arrangement was investigated (Adrian, 1925) by maceration and teasing, shows endplates in scattered bands at various levels along its length. The macerating agent used was 20% nitric acid, and after 24 hours - the duration of maceration in Adrian's work - the muscle fibres must have undergone considerable shrinkage; in the course of thepresent work, attempts were made to macerate muscles in 20% nitric acid, and a shortening to about two thirds of the original length was produced. Adrian's results are none the less of some value in this argument. He found that from a 14 cm. long muscle, the average length of unbroken fibres was 17.3 mm., and while he admits that longer fibres might have been originally present, the fact that the longest intact fibre obtained was 27 mm. long, indicates that this muscle is, as Adrian claims, built up of fibres about 2 cm. long arranged in series. Adrian quotes Eisler (1912) to the effect that this arrangement is common in long muscles.

Thus it appears that some short muscles in the rabbit and cat possess fibres which extend right through the muscle, and these have a single band of motor endplates. Other, larger muscles, having a scatter of endplates, are built up of

several relays of interdigitating fibres. This is further supported by the observations of Clark (1931) in his studies on the size of motor units. Discussing the difficulty of finding out the total number of muscle fibres within a muscle, Clark points to the inadequacy of cross sectional fibre counts in many muscles on account of the fact that some muscle fibres may not extend through the whole length of a muscle and may therefore be missed in some cross sections. Clark (1931), following Denny-Brown (1929), describes the soleus muscle of the cat, and points out that every fibre runs from end to end of its fasciculus. In the extensor digitorum of the cat, however, a certain proportion of the individual fibres taper off in the substance of the muscle, the average length of a short fibre in Clark's investigations being 11.5 mm., as against a total fasciculus length of 16.4 mm.

There remains the possibility that some fibres in muscles with scattered endplates may be plurally innervated.

The two limb muscles from a human foetus studied differ in their endplate distribution. In biceps humeri, the endplates are in a single band round the middle of the body of the muscle, whereas in sartorius, each fasciculus possesses several groups of endplates along its length. Schwarzacher (1959) has studied a number of human muscles from foetuses, children and adults, with the acetylthiocholine technique, and obtains the same results from these two muscles. In addition, he observes that all the human muscles in his

series possess a single band of endplates, with the exception of gracilis (and sartorius). In gracilis, Schwarzacher finds two or three clusters of endplates along the length of fasciculi, and in sartorius, five or six such endplate groups. By isolating individual fibres from these stained fasciculi, he finds that these are formed of a series of overlapping muscle fibres lying end to end. Nearly all the isolated fibres have a single motor endplate situated near the midpoint of the fibre, and from this it would seem that the distribution of endplates in a muscle is a definite guide to the architectural arrangement of its component fibres.

Schwarzacher's results on the human sartorius conflict with those of Lockhart and Brandt (1938) on the human sartorius; these workers were able to isolate a fibre of 34 cm., with both ends broken, from a human sartorius 52 cm. long. In a foetal sartorius 5 cm. long, they state that the fibres could be shown, by teasing, to extend from end to end of the muscle.

These observations on endplate distributions in mammalian muscles suggest that, for any one species, while the smaller muscles may be built up of fibres running through the whole muscle, certain of the larger muscles are composed of relays of interdigitating fibres. Thus, in the rat, only the longest muscles such as anterior gracilis and sterno-hyoid have two bands of endplates. In the rabbit and cat, the

the short-fibred jaw muscles have one band, but many larger muscles have a scattered distribution which, in the few cases studied, generally indicated that these muscles are composed of relays of interdigitating fibres.

It appears to be the size of a muscle relative to other muscles in the same animal, rather than the absolute size of the muscle itself, that decides whether a muscle is built up either of individual fibres which extend from end to end, or of relays of fibres. Thus, in the rat the muscles with two bands of endplates are the longest ones in the body, all the shorter muscles having a single band. Muscle fibres in the diaphragm of the rabbit, which is in part of its extent a single-band muscle, are comparable in length with the longest muscles of the adult rat. In a full-sized rabbit or cat, the longest muscles have liberally scattered groups of endplates, yet they may be many times shorter than human muscles which have a single band. In man it is only the two longest muscles, gracilis and sartorius, which have been shown to have a scattered distribution of their motor endplates.

3. Evaluation of the Present Combination of Techniques.

Using the old-established histological techniques employing silver, gold, and methylene blue, the earlier workers demonstrated nerve endings on muscle fibres often with considerable elegance and precision. The preparations illustrated by Boeke (1921) using the Bielschowsky technique are an outstanding example of this. In many cases, however, the functions of the endings described by older workers remained in doubt unless the central connections of the nerve fibres could be traced, and results were frequently misinterpreted. It is only when these older methods are supported by coincident investigations using modern techniques such as cholinesterase localisation and electron microscopy, that the true significance of many findings is realised.

An example of this may be found in one of the most primitive forms of vertebrate neuro-muscular junction, the so-called 'basket ending', which is found at the ends of segmental muscle fibres in a number of lower vertebrates. These endings were described in detail in 1898 by the Italian, Giacomini, in the myotomes of certain fish and Amphibia. Giacomini considered these endings to be sensory, and this view was supported by Perroncito (1902) and later workers, so that as recently as 1953, Tiegs believed them to be stretch receptors. That these endings are in fact motor terminations was only determined when the newer techniques were applied to their study in Amphibia. Cholinesterase

staining shows that the enzyme is only found at the ends of the muscle fibres (this was first shown by Lewis and Hughes, 1957, 1960; and Lewis, 1958); and this observation is supported and extended by electron microscopy which reveals the detailed position and structure of the nerve endings. It shows the poorly developed subneural apparatus deep to the endings, and this is suggested as a possible reason for the diffuse cholinesterase reaction, the enzyme being perhaps less efficiently localised than in the motor endplates of higher vertebrates where the electron microscope has revealed a complex subneural apparatus, and where cholinesterase is seen in suitably stained preparations (e.g. Couteaux, 1958) outlining the gutters of this apparatus.

4. Cholinesterase at Muscle-Tendon Junctions.

The existence of a cholinesterase reaction at muscle-tendon junctions was discovered independently and within two months by Couteaux (1953) in muscles of the frog and mouse, and Gerebtzoff (1954) in the rat. It has since been demonstrated in the mouse, rat, guinea-pig, rabbit, cat, dog, and in man (Gerebtzoff and Ueten, 1954); in the goat (Beckett and Bourne, 1958); in birds (Gerebtzoff, 1955; Bonichon, 1957); and in a number of lower vertebrates (Gerebtzoff, 1960), including the sea-horse (Couteaux, personal communication), In the present studies it has been reported in the lamprey, dogfish, Amphibia, and reptiles, and was also seen in Myxine, in the limb muscles of a salamander, and in the mouse sartorius.

It is apparent that cholinesterase is of normal occurrence at muscle-tendon junctions. That it is true acetylcholinesterase has been shown by Schwarzacher (personal communication), who estimates that the amount present is from 20% to 35% of the quantity at the neuromuscular junction. It has been shown (Beckett and Bourne, 1958) to appear at two-thirds term in the foetal goat, and this is considerably later than the time of appearance of the motor endplates. The same workers report its occurrence in the human foetus.

Although afferent nerve terminations have been demonstrated in the region of muscle-tendon junctions, the enzyme appears to be independant of these since it persists unchanged even

240 days after nerve section (Gerebtzoff and Ueten, 1954; Gerebtzoff, 1957). Observations on the effect of tenotomy (Reznik and Gerebtzoff, 1956; Reznik, 1956; Gerebtzoff, 1957) are of interest. A clean section of tendon produces no effect of the enzyme, but if repair of the tendon is hindered, the enzyme slowly increases from 8 to 20 days after the operation, reaching its maximum level about 40 days after tenotomy and falling to normal again some 2 to 3 months after the original procedure. The same effect follows section of either the proximal or distal tendon, or transection of the whole muscle mass. If most of the muscle mass is resected, however, activity falls rapidly. Schwarzacher (personal communication) has confirmed these effects of tenotomy, and has observed persistence of the enzyme several weeks after denervation.

The architecture of the muscle-tendon junction has been described in detail by Schwarzacher (1960) and Muir (1960), and has been illustrated in this work for Amphioxus, lamprey, dogfish, goldfish, and Xenopus. It is characterised by a series of clefts, produced by invagination of the sarcolemma into the ends of the muscle fibre. It is known that as well as being present at sites of motor innervation, cholinesterase is also present in much lower concentration throughout the whole of the muscle fibre (Snell, personal communication). If this latter cholinesterase is related to the sarcolemma, the myotendinous reaction might be due to the very great increase in sarcolemmal surface area which occurs at this region.

5. Subdivision of Skeletal Muscle Fibres into Two Types.

Many criteria have been advanced for the subdivision of skeletal muscle fibres into two distinct types, and a selection of illustrative examples will now be cited.

a. Naked Eye Appearances :

Starling (1920) pointed out that the white pectoral muscles of the domestic chicken contrast with the dark red of these muscles in the goose, duck, and pigeon, or with those of birds which are relatively indefatigable in flight like the buzzard or falcon. It was pointed out in the present work that the red semitendinosus of the rabbit lies within the substance of the white adductor magnus, an observation made in 1873 by Ranvier, who described differing histological characteristics of the two muscles. Meyer (1875) pointed out, however, that certain red muscles in the rabbit, such as the flexor digitorum communis and the masseter, show the histological characteristics described by Ranvier in the white muscle.

Differences in colour between muscles in lower vertebrates have also been noted. Hines (1927) states that frog muscles are all white in colour apart from the superficial muscles of the neck. Breakann (1956) describes a red muscle section in the middle of the white muscle in several Teleosts and in the Porbeagle shark, and Cole (1907) refers to red and white muscle fibres in Myxine.

The reason for the redness of some muscle was studied by Kuhne (1865) who examined the absorption spectrum of minced

extracts of red muscles from which all the blood had earlier been washed out. He observed the presence, in this red muscle, of a pigment which he identified with haemoglobin, and Lankester (1871) associated the presence of haemoglobin with muscles where prolonged activity was required. Keilin (1925) demonstrated that the haemoglobin in red muscle from the pigeon differed slightly in its absorption spectrum from the haemoglobin of blood, and it is now established that the degree of redness of muscle depends on its content of muscle haemoglobin, or myoglobin (Needham, 1926; Millikan, 1939; Biorck, 1949). Denny-Brown (1929), however, believed that the red pigmentation is not essential to the slow type of contraction, and is probably "the outward sign of some function not closely related to contraction", and Walls (1953) found little difference in the myoglobin content of the human soleus and gastrocnemius, which he stated to be red and white respectively.

It seems to be established, then, that many muscles are red in colour and that this redness is the result of the presence of myoglobin. Observations such as those of Meyer (1875) and Walls (1953) could be explained by the statement of Starling (1920) that all striated muscles of higher vertebrates contain both types of muscle fibre, and that few muscles are almost exclusively red or white.

b. Histological Structure :

It was shown by Bowman (1840) and by Mayeda (1890) that there

is a considerable range in the diameter of skeletal muscle fibres for any particular species. Variation may also be seen in cross sections through individual muscles, and while this may to some extent be due to tapering ends of fibres ending within the muscle, there is evidence that two distinct calibres of fibre may be present in the same muscle. This has been shown to be the case in muscles from the dog, quail, and starling (Bosiger, 1950); pigeon and bat (George and Jyoti, 1955); fish (Boddeke et al, 1959); and in the cockroach (Smit, 1958). Many other instances could be cited. While many muscles are built up of mixtures of the two calibres of fibre, some have only one or other type; thus the pectoralis major muscle in the pigeon possesses both broad and narrow fibres, the narrow fibres being sixteen times as numerous as the broad fibres which are confined to the periphery of the muscle (George and Jyoti, 1955). However, the pectoralis major in the parakeet and the bea-eater is made up of broad fibres only, while in the domestic folw and the kite, only narrow fibres are present (George and Naik, 1958).

Ranvier (1873, 1874) pointed out that red muscle fibres possess relatively more sarcoplasm than white fibres, and Knoll (1891) speaks of "protoplasm poor" and "protoplasm rich" fibres. Hines (1927) agrees that there is more sarcoplasm in red fibres than in white, but Walls (1960) states that while on the whole red muscle contains more sarcoplasm than white, this is not always so, and red fibres

may possess little and conversely white fibres may possess much sarcoplasm.

A number of workers have commented on the situation of the nuclei in the two types of fibre. Ranvier (1873, 1874) speaks of the nuclei in red fibres being more numerous and situated within the depths of the fibres as well as beneath the sarcolemma, and Hines (1927) states that nuclei in red muscle are not always immediately beneath the sarcolemma, as is true for white muscle.

The granular appearance of the sarcoplasm has been commented on by many of the older workers. Meyer (1875) speaks of the finely granular sarcoplasm of red muscle fibres, and states that when there are many granules, fibres appear dark in section. The subject is reviewed by Bullard (1912), who states that white muscle may have fibres which appear just as granular as those of red muscle. Bullard also considers the nature of these granules, and concludes that they may be divided into the "true interstitial granules" described by Kolliker (1857), and fat droplets. The "true interstitial granules" have since been identified as mitochondria (Wachstein and Meisel, 1955).

Ranvier (1873, 1874) discussed the differing appearances of the striations in red and white muscle, and stated that red muscle fibres are more distinctly striated longitudinally, but that their transverse striation is less regular. Schafer (1893) reported for man that the clear sarcoplasmic or white

fibres contain small myofibrils arranged rather regularly, while in the granular sarcoplasm of red fibres, the myofibrils are large and the arrangement without order.

The sarcomere length (distance between two adjacent Z bands) was shown by Jasper and Pezard (1934), and by Szekessy (1947), to be short in muscles of Crustacea and Insects which contract rapidly, and long in muscles with a slow contraction. Boddeke (1959) has shown in fish that broad muscle fibres have short sarcomeres relative to those of the slender muscle fibres.

The capillaries in the red muscle of the rabbit were shown by Ranvier (1873,1874) to run a more tortuous course than in white muscle, with frequent dilatations of the crosss/connecting vessels. Bosiger (1950) found that slender muscle fibres in the quail have 20 to 40 times as many capillaries per unit of fibre surface, as do the broad fibres, and Boddeke et al (1959) found that the narrow muscle fibres of the tench have 12.5 times as many capillaries per unit of fibre surface as the broad fibres.

Kruger, (1929, 1952) distinguishes between two varieties of skeletal muscle fibre on the basis of their cross-sectional appearance. His observations have included many species of bird, and the frog where his findings have been confirmed by Gray (1958). More recently (1957) Kruger has extended his studies to mammalian muscle, and has described these two varieties of fibre in the rat. His claim is that one type of fibre, which he equates with the

slow fibre in the frog, has no organisation of its component myofilaments into groups to form myofibrils, and in cross sections the contractile material appears to be arranged in irregular, large fields; this form he terms "Felderstruktur". The second type, in which the myofilaments are separated into groups to constitute myofibrils, show on cross section a "Fibrillenstruktur", and correspond to the fast or twitch fibre in the frog.

c. Fine Structure :

Electron microscopic observations have also been advanced to distinguish between two groups of skeletal muscle fibres. Edwards et al (1956) have studied a series of muscles from Crustacea to man, distinguishing between high frequency and low frequency muscles. They state that there appear to be two, easily distinguishable, general categories of striated muscle structure. The high frequency muscle is characterised by widely-spaced, non-branching fibrils of large diameter and short period, little endoplasmic reticulum, and considerable numbers of large mitochondria; this muscle is richly vascularized, has high oxidative activity, and is dark in colour relative to other muscles of the same species. Low frequency muscle is in general relatively poor in sarcoplasm and mitochondria, and has a relatively long period. They point out, with regard to the colour of muscles, that the high frequency muscles of insects are reddish and the low frequency muscles pale, whereas in

vertebrates the reverse is true.

Ruska (1958) has shown with the electron microscope that the anterior latissimus dorsi of the crow does not have its myofilaments separated into bundles, as in the posterior latissimus dorsi where myofibrils are evident.

d. Innervation:

The investigations of Kuffler (1953) have shown the existence in the frog of two distinct nerve-muscle systems, each consisting of a separate set of skeletal muscle fibres which are innervated by a special group of nerve fibres. In one, the muscle fibres give fast, or twitch, contractions, and are supplied by large diameter nerve fibres, while in the other, the muscle fibres are slow in their response, and are supplied by small diameter nerve fibres with consequently lower conduction velocity. Whereas the slow muscle fibres are densely innervated by small nerve fibres many of which extend all along their length, the twitch fibres have at the most only a very few endplates and these are relatively widely spaced. Kuffler states that there is no overlap in innervation between the two systems.

Similar findings have been reported in avian muscle by Ginsborg (1959), who reports that muscle fibres of the chick are innervated in one of two ways. One type of fibre is supplied by a single axon and has a focal endplate, while the other variety is supplied by a number of axons and has neuromuscular junctions at many points along its length.

Unlike the Amphibian slow fibres, however, the chick fibres with a diffuse innervation were found to respond to adequate stimulation with propagated action potentials.

Kruger (1952) has shown that the slow Amphibian fibres have motor neuromuscular junctions of the 'en grappe' type, while typical endplates are present on the twitch fibres. The differing cholinesterase patterns on these two types of fibre have been illustrated by Couteaux (1955).

e. Biochemical and Histochemical Observations :

Breakkan (1956) has shown that red muscle fibres in fish have a much higher fat content than white fibres, and that they contain relatively higher concentrations of the B group of vitamins and of cytochrome. These findings were confirmed in a number of Teleosts by Boddeke et al (1959), who also found that glycogen was present in both types of fibre, whereas George and Naik (1958) found glycogen in the broad fibres only. George and Naik (1958) found a large amount of fat in the red muscles of fish, and likewise in the narrow, red flight muscles of the dove, where no fat was detected in the broad, white fibres.

Succinic dehydrogenase was shown by Thimann and Padykula (1955) to be small in amount in white muscle fibres of the rat, whereas red muscle fibres gave a uniformly strong reaction. Nachmias and Padykula (1957) were able to differentiate red muscle fibres in the rat from less granular fibres with paler sarcoplasm, by histochemical

methods for demonstrating succinic dehydrogenase, lipids, and esterase.

6. Indications from Results of Present Work that Skeletal Muscle Fibres may be Subdivided into Two Types.

From the examples given in the previous section, it can be seen that there is considerable justification for the subdivision of skeletal muscle fibres into two distinct types, but that the distinction is not clear cut, and that fibres of one or other group may differ in certain characteristics from similar fibres in a different species.

The results of the present work provide a number of examples of two types of muscle fibre in one species, and it will finally be considered how far these two types display corresponding features in different species.

In Amphioxus, only one type of muscle fibre was observed, but the muscle units of the lamprey contain two distinctly different varieties of fibre, the parietal, arranged as fibres, and the central plates, which form flat sheets of muscle. It was pointed out in the description of these two forms of muscle, that they differ in their histology as observed both at the light microscope level and with the electron microscope, as well as in their respective patterns of cholinesterase distribution and the positions of their neuromuscular junctions. Thus, with the light microscope it was observed that the parietal fibres have on cross section a looser texture than the central plates, that they are well supplied with nuclei, and that they are associated with a rich plexus of nerves. With the electron microscope it was seen that the amount of

sarcoplasm in the parietal fibres is greater than that present in the central plates, and that they are richer in mitochondria. Cholinesterase is found only at the ends of the central plates, whereas it is present all along the parietal fibres, and electron microscopy shows that motor neuromuscular junctions are present in this distribution. The parietal fibres are thus associated with nerves all along their length, and in this respect they resemble the slow variety of muscle fibre described by Kuffler (1953) in Amphibia, and the multiply-innervated avian muscle fibres which have been demonstrated histochemically in the present work. The central plates of the lamprey, which are innervated only at certain focal points, seem to correspond with the amphibian twitch fibres, and with focally innervated avian fibres.

Support for the functional association of these two types of fibre in the lamprey and amphibia is provided by recent work of Andersen and Jansen (personal communication), who have demonstrated the two types of muscle fibre in Myxine histologically and observed the distribution of sites of cholinesterase in relation to them. The electrophysiological experiments of these workers show that the parietal-like and central plate-like fibres in Myxine give respectively slow and fast responses to stimulation.

Two calibres of muscle fibre were observed in myotomes of the dogfish. The broad fibres, which predominated, were innervated paraterminally, while the peripheral, narrow fibres were innervated at varying points along their length.

Boddeke et al (1959) show that two calibres of muscle fibre may be found in the myotomes of many species of fish, and Breakkan (1956) tried to associate the amount of the slender variety of fibre with the behaviour of the fish. These workers conclude that slender muscle fibres are more plentiful in fish requiring a large staying power, and George and Naik (1958) point out that the large fat content of these fibres is indicative of their prolonged activity, the fat being used as fuel.

Only one form of muscle fibre was observed in the myotomes of Amphibia, the innervation being exclusively by basket endings. Similarily, in the segmental muscle of the two reptiles studied, only one variety of muscle fibre and innervation was seen in each species. That two varieties of muscle fibre do occur in Amphibia is established by the work of Kuffler (1953), but the fact that these are found only in the adult suggests that they may only emerge once a terrestrial existence has been assumed. It would be interesting, in this connection, to know if more than one form of muscle fibre is present in the limb muscles of Urodeles. Certainly in these muscles a wide range of innervation forms may be observed, as was pointed out by Mather and Hines (1934) in their studies on the limb muscles of the Oregon newt.

In the work on avian muscle, it was shown that two types of muscle fibre could be demonstrated histochemically in the chick. One type of fibre had many neuromuscular junctions along its length, and the other had one, or at the most,

very few endings. In most of the muscles studied, both types of fibre were present, but the anterior latissimus dorsi appeared to consist entirely of fibres having many motor endings. In their mode of innervation, therefore, these multiply-innervated fibres of the chick resemble the twitch fibres of the frog, but, as was pointed out in the previous section, the fibres of the two species differ in their physiological response to stimulation.

The resemblance between the chick and frog multiply-innervated fibres is supported by the pattern of cholinesterase distribution at endplate sites, which in the anterior latissimus dorsi was observed to be similar to that of the frog slow fibres (Couteaux, 1955, and personal communication), and the resemblance goes further in that there are roughly the same number of endings on the two fibres. Thus, some fibres in the anterior latissimus dorsi have about eighty endings, and this corresponds with a figure of over seventy reported from the ilio-fibularis of the frog by Couteaux (see Burke, 1956).

The slow fibres of the frog have been stated by Kruger (1952) to have a "Felderstruktur", and the present results suggest that the multiply innervated fibres in avian muscle may be identical with the avian fibres which Kruger (1950) describes as having a "Felderstruktur". The most direct evidence in favour of this identification is the finding

that in the pigeon the latissimus dorsi consists largely, if not exclusively, of fibres with multiple neuromuscular junctions, since this muscle is amongst those found by Kruger to consist entirely of fibres with "Felderstruktur".

Studies on the Growth of Skeletal Muscle Fibres

It is known that an adult muscle grows to many times its length at birth, and much if not all of this post-natal longitudinal growth is the result of increase in length of the component muscle fibres. The object of these studies is to determine the site, or sites, on a skeletal muscle fibre at which growth in length takes place.

Schmidt (1927), in a long paper on the histogenesis of muscle fibres, surveys the literature on the subject up to that date. A number of other early workers are cited by Crawford (1954). All believed that the area of sarcoplasm between the terminations of the myofibrils and the attachment of collagen to sarcolemma, was the site of formation of contractile tissue. Spiedel (1934) observed the muscle fibres in the tadpole tail, directly, over prolonged periods of time, and came to the conclusion that the site of active growth in length is this undifferentiated zone of protoplasm at the muscle-tendon junction.

The subject has been studied experimentally by Crawford (1954), who investigated growth in the tibialis anterior muscle of the rabbit, marking the muscle belly at intervals, either with indian ink spots on the surface, or by inserting loops of tantalum wire; the second method possessed the advantage that the positions of the wires could be followed by x-raying, so that a number of results could be obtained before the animal was sacrificed.

Both methods used by Crawford showed more or less even separation of the markers, but as he pointed out, while this indicates that interstitial growth occurs, the same conclusion may not be applied to the individual muscle fibres themselves, since these may not extend through the whole length of this muscle; its interstitial growth could therefore result from growth at the ends of the component fibres and some rearrangement of their position.

In an earlier section of this work, the distribution of motor endplates in the rabbit tibialis anterior is described. These are found in broken and irregular bands at several levels in the muscle, and a similar distribution is found in the rabbit sartorius, which has been shown by Van Harreveld (1947) to be made up of relays of muscle fibres each shorter than the total length of the muscle. It may well be, then, as Crawford suggests, that the muscle fibres in the tibialis anterior do not extend from one attachment of the muscle to the opposite attachment.

The muscles of the rat have, however, been found to possess relatively simple arrangements of their component muscle fibres, and in the present studies, growth of these muscles has been studied with tantalum wire markers.

Methods

Rats were operated on when three to four weeks old. At this age the muscles are of sufficient size to retain the wire inserts, and considerable growth in length occurs

in a uniform manner, the loop types presented certain theoretical disadvantages. A tight loop will constrict the muscle fibres round which it passes as these increase in girth, and a loose loop would be liable to tangle with surrounding structures which might impede its movement, and also by its mobility would make it difficult to find a fixed point from which to take measurements. Similarily, the strip of wire bent round the edges of a muscle might constrict the fibres and foul surrounding structures. The type of marker consisting of a single short strip of wire with a knot in the middle, completely immersed within the muscle, was selected and used in subsequent experiments.

Results

In the initial series of experiments, eleven rats were used, and in each, two wire markers were implanted in the anterior gracilis of the right leg, and two in the sterno-hyoid. The animals were x-rayed shortly after the markers had been introduced, and thereafter at weekly intervals until they were fourteen weeks old and had undergone their period of most rapid growth. The markers were inserted transverse to the long axis of the muscle, but as growth took place some tended to swing round till they lay longitudinally.

Results were charted in two ways. X-rays (on small dental films) were placed between two sheets of glass and projected with a photographic enlarger onto sheets of paper. Prior to

this, the positions of the muscle attachments had been marked on the x-ray with ink or by scratching the emulsion, and they could then be seen clearly on projection. A base-line was drawn on the paper, and the origin attachment placed on this. Magnification was kept constant, and in each case the positions of the markers and the insertion attachment were marked on the paper. This was done for each of the weekly x-rays, and the results graphed. It was seen, by the time the animals had reached the age of about ten weeks, that the changes taking place in the positions of the markers were fairly regular, and therefore that weekly x-raying was unnecessary. One final film was therefore taken four weeks later, to end the series.

The results (Plates 4 - 9) showed that the markers were separating fairly evenly from one another and from the ends of the muscles. The lines connecting the markers on the graphs are fairly straight, but now and again there is an apparent anomaly where the total length of the muscle on one reading is less than that on the previous reading. The length of the muscle is affected by flexion or extension of the joints of the part, even when the animal is relaxed under chloroform as was the case when the films were taken.

These irregularities do not show if the x-rays are projected so that the two ends of the muscle are a constant distance apart - 10 cm. was arbitrarily chosen as the distance - and the positions of the markers indicated in relation to the ends of the muscle. If the markers are separating evenly

from one another and from the ends of the muscle, they will on this second type of projection remain at constant distances from one another and from the muscle attachments. This did appear to be the case, though the mobility of the loop type of marker, and variations in the points on the lines of muscle attachment from which measurements were taken, produced irregularities. The most consistent results were obtained from the small type of marker completely immersed within the muscle substance, and this type of marker was used in all subsequent experiments (Plate 9).

Twenty-four rats were then taken, twelve males and twelve females, and in half the anterior gracilis was used, and in half the sterno-hyoid. Four markers were placed in each muscle. The rats were operated on when three to four weeks old, and were x-rayed shortly after the introduction of the markers, and then again on two occasions at intervals of four to five weeks. The results were recorded by the second method described above, where the muscle ends are brought to a constant distance apart in projection. Instead of marking the whole length of the muscle attachments on the films, the midpoint only was represented.

Results (Plates 10 - 17) again show that the markers are separating evenly from one another, maintaining their positions relative to each other and to the ends of the muscle.

After these experiments had been carried out, it was found that sterno-hyoid has a second band of endplates deep to the

sternum, and dissection, together with examination of serial cross-sections of the lower part of the muscle, showed that in its lower extent it is no longer a strap muscle; the outer fibres curve forwards and inwards, anterior to the inner fibres, so that the muscle converges on an attachment to the narrow, lower part of the manubrium, rather than being inserted as a strap to the back of the circular part.

Sterno-hyoid could thus not be taken as an example of a single-band muscle, but its companion in the neck, sterno-mastoid, which could be seen in its whole length, showed one single band of endplates at its middle, and as has been described in an earlier section of this work, counts of fibres in cross sections at intervals along the muscle indicate that the fibres run from one attachment to the other. This muscle was therefore taken as an example of the single-band type, and four rats were operated on, under the same conditions as before, both sterno-mastoids being used in each animal and four markers being inserted in each muscle. Again (Plates 18 - 21) the markers were found to separate proportionately from one another and from the ends of the muscle.

Since this method of charting results gives no indication of the amount of actual growth of the animal, the length of the femur was taken in those rats in which the anterior gracilis had been used, as an indication of increase in size of the animal. For the neck muscles, the actual length of the muscle was taken. These measurements are shown with the results of the x-ray projections. In one instance for each

of the three muscles used, the actual x-rays have been employed to show the markers separating (Plates 12, 16, 18).

Results from all three muscles are very similar. The markers separate evenly from one another, maintaining their positions relative to the ends of the muscle. Often they change position slightly between the first and second monthly x-rays, whereas positions of the markers at the third are usually very similar to those at the second. Occasionally a slight apparent drift of all four markers to one end of the muscle is noticed, but this is always slight compared with the overall changes in length that have taken place, and the markers invariably maintain their relative distances from one another. Though the wires were inserted transverse to the long axis of the muscle, they did in some cases rotate, even to the extent of coming to lie in the same line as the muscle fibres. The central knot on a wire was, however, assumed to have remained in its original position through any rotation that occurred.

An attempt was made to study the relation of the wire markers to the muscles, the intention being to remove the wires just before embedding the muscle in paraffin, and then to section the muscles longitudinally in order to study the positions of the wire tracks in relation to the muscle fibres. The wires were, however, found to be firmly adherent to the muscle substance, and on pulling them out, tears were produced in the muscle. This project therefore had to be abandoned.

Discussion

1. Significance of Results with Wire Markers.

The muscles of the rat are chosen for these experiments because of their simple structure. The object of this study is to determine at what point, or points, along its length a muscle fibre grows. If the arrangement within a muscle of its component muscle fibres is known, and if these muscle fibres can be marked at points along their length and the markers followed throughout a period of growth, it should be possible to form conclusions regarding the site, or sites, of growth.

The muscle fibres in the rat sterno-mastoid extend from one attachment of the muscle to the other. By placing four markers within the muscle and following their separation over a period of three months, it was hoped that a definite indication of the growth site, or sites, would emerge. If, as the older workers believed, the sarcoplasm at the muscle-tendon junction is the active growth site, it would be expected that the markers would gradually move relatively further and further from the muscle ends. If, on the other hand, growth takes place all along the fibre, the markers should retain their positions relative to one another and to the muscle ends.

As has been described above, the markers remained at proportionate distances from one another and from the attachments of the muscle. This was also the case in the rat sterno-hyoid and anterior gracilis. These findings

indicate interstitial growth.

It remains doubtful, however, whether the markers indicate growth of the muscle fibres themselves. They may equally well signify changes taking place in the connective tissue of the muscle. It is unlikely that any muscle fibre could survive for a length of time after having been transfixed by a piece of tantalum wire, and most probably these wires are embedded in the perimysium surrounding the muscle fibres. Unfortunately it is not possible to remove the wires to ascertain their actual position relative to the muscle fibres and connective tissue.

2. General Discussion on Muscle Fibre Growth.

There are several possible sites for growth in length on a skeletal muscle fibre. Older workers were of the opinion that growth took place by the accretion of new sarcomeres at the ends of the muscle, in the region of the muscle-tendon junction, and this view was supported by Spiedel in his studies on the living tadpole tail, where counts of the numbers of sarcomeres showed that they increase progressively as growth occurs.

Growth might, however, take place all along the muscle fibre - regular interstitial growth, such as was suggested by the movements of the wire markers in the present studies. Growth could conceivably be confined to certain points along the shaft of a fibre, such as the middle section, or the segments opposite the fibre nuclei.

There is no evidence of any cytoplasmic differentiation along the shaft of a muscle fibre. Comparison of the length of sarcomeres in young and adult animals shows that there is little change between the two ages, yet the muscles may more than double their lengths in the interval. Growth in length must therefore be, as Spiedel stated, by the addition of new sarcomeres rather than by the expansion of those already present. This could take place all along a muscle fibre, thereby producing interstitial growth, but it is unusual to see variation in sarcomere size, other than the alterations produced by degrees of contraction of the myofibrils (though short sarcomeres have been reported in tissue cultures of developing muscle). Certainly, short sarcomeres are a rarity, and examination of a skeletal muscle fibre does not show the range in size of sarcomeres that would be expected if these were being developed all along its length.

Moreover, the biochemical composition of a sarcomere probably demands a constant number of cross linkages between the actin and myosin filaments (Huxley, 1957), for each individual species at least, and therefore interposition of "baby" sarcomeres is unlikely.

The most feasible site on a skeletal muscle fibre for accretion of new sarcomeres remains the zone of protoplasm between the termination of the myofibrils and the attachment of the tendon to sarcolemma. This was the view of Schmidt and earlier workers, since supported by Spiedel, and made no less feasible by the recent electron microscopic findings on

the ultrastructure of the myotendinous junction.

If it is the case that the ends of the muscle fibres are the sites of active growth, and the markers used in this series of experiments are growing with the connective tissue, then we must envisage a growing skeletal muscle fibre sliding as it lengthens within its surrounding tube of connective tissue.

The motor endplate is built into the side of the muscle fibre, the terminal ramifications of the nerve indenting but not penetrating the sarcolemma. It seems hard to believe that so highly organised a structure as the endplate could alter its position in relation to the contractile substance of the muscle fibre as it grows, but if growth takes place at the ends of the myofibrils only, then the motor endplate must move.

In single fibres isolated from the sartorius muscle of an adult frog (personal observation), two endplates have been demonstrated histochemically, with the interval between them greater than the total length of the limb in a small animal of the same species. Similarily, in the anterior latissimus dorsi muscle of the chick, the endplates have been shown to separate proportionately with the increase in length of the muscle as a whole. These changes could only come about in one of two ways; either the motor endplate alters itsposition on the growing fibre, or the fibre grows interstitially.

3. Possibilities for Future Study.

a. Nuclear Studies :

Schmidt and others have emphasised that the muscle nuclei play an active part in growth of the myofibrils, and Crawford refers to rapid nuclear proliferation occurring in the regions of undifferentiated sarcoplasm at the fibre extremities. If this be the case, it should be possible to discover the sites of active growth by determining the positions of concentrations of actively-dividing nuclei. These might be shown by arresting mitosis with colchicine, or by indicating dividing nuclei with radioactive thymidine.

b. Physical Labelling of Muscle Fibres :

This is technically difficult to accomplish on account of the small diameter of muscle fibres, and any damage to a fibre is liable to kill it or at least to distort its growth processes. A scar sufficiently slight to allow a fibre to continue living and growing will be difficult to detect again, and it would be virtually impossible to mark the same fibre in more than one place. A point on a fibre might be marked by depositing iron ions from an electrode, its position subsequently being sought by means of the Prussian blue reaction, but again the deposition of sufficient iron to give a clearly visible reaction would in all probability kill the fibre.

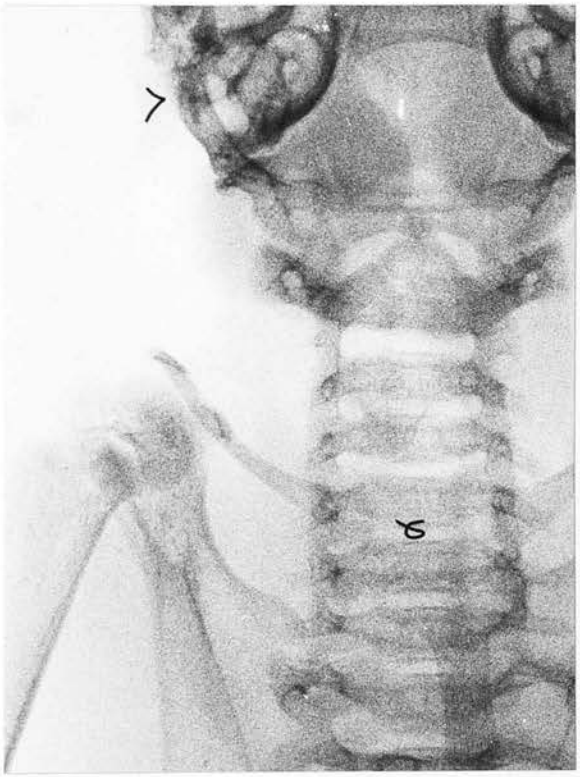
Fluorescin iso-cyanate, which attaches itself to proteins and can be detected in sections with the fluorescence microscope, might provide the answer. If a quantity were

injected into the substance of a muscle, a portion would presumably attach itself to the connective tissue, while some would penetrate the muscle fibres themselves and grow with them. The distribution of the substance could then be detected after varying periods of growth, by cutting thin sections and examining them by fluorescence microscopy.

c. Synthetic Labelling of Myofibrils :

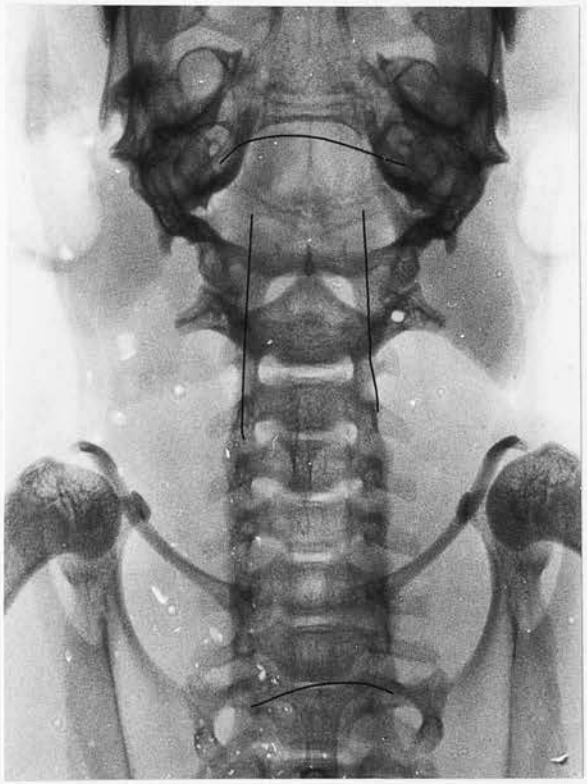
The site of formation of new sarcomeres might be shown by labelling an animal's amino acid pool with a radioactive amino acid essential to the muscle proteins. The positions in the myofibrils of this substance could then be detected by radioautographic techniques.

Rat - young adult showing motor endplates in muscles of neck.
Acetylthiocholine.
(x 2 approx.)



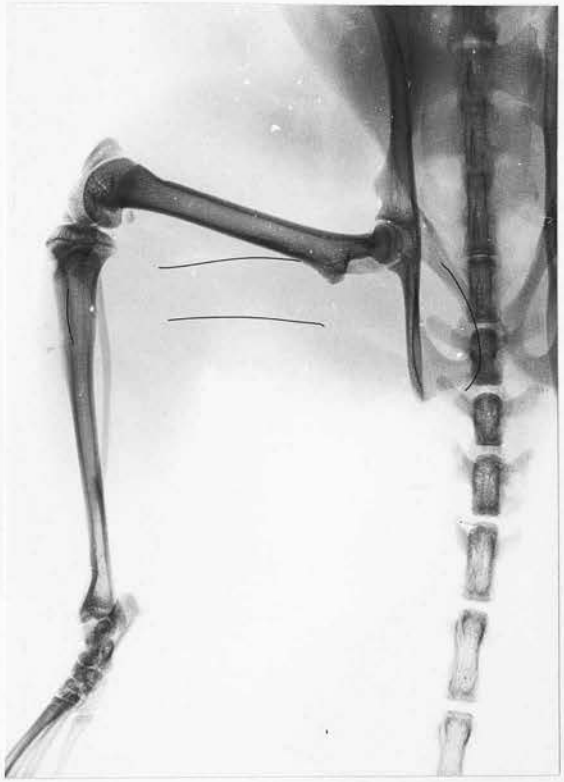
Rat - x-ray of young adult with wire inserts near sites of attachment of right sterno-mastoid.
(x 2 approx.)

Rat - young adult showing motor endplates in muscles of neck : a window has been dissected from upper part of sternum to expose second band of endplates in sterno-hyoid.
Acetylthiocholine.
(x 2 approx.)



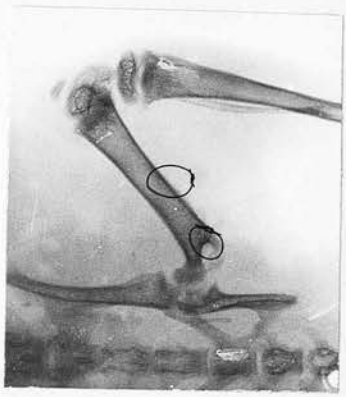
Rat - x-ray of young adult showing wires inserted near attachments and down margins of sterno-hyoid.
(x 2 approx.)

Rat - young adult showing motor endplates in muscles of medial aspect of right thigh.
Acetylthiocholine.
(x 2 approx.)



Rat - x-ray of young adult in which wires have been inserted near attachments and down margins of anterior gracilis.
(x 2 approx.)

Anterior Gracilis 1



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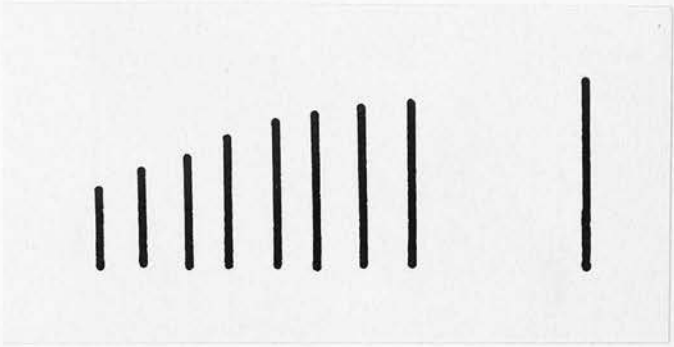
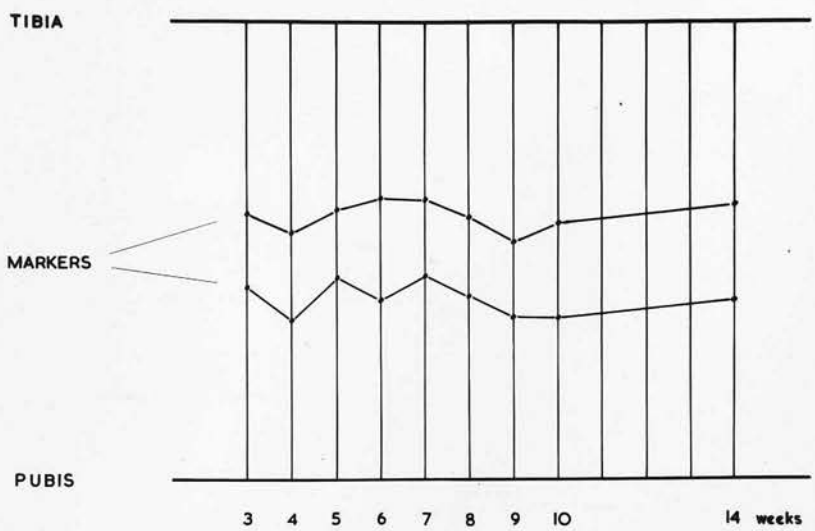
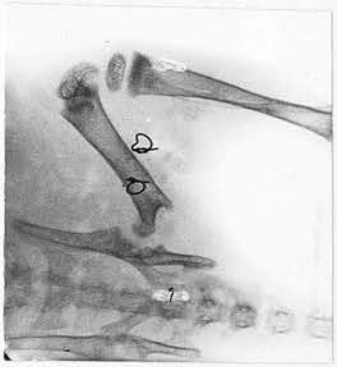


Positions of Markers



Anterior Gracilis 2



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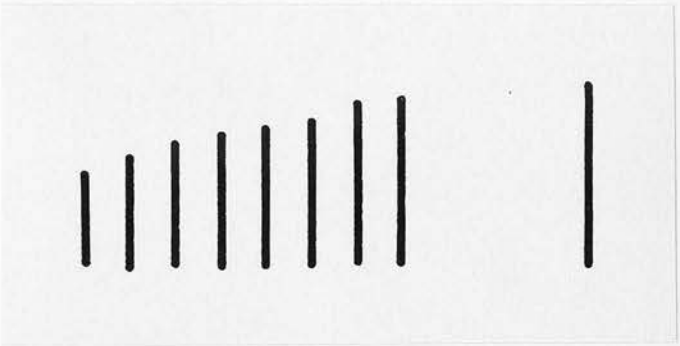
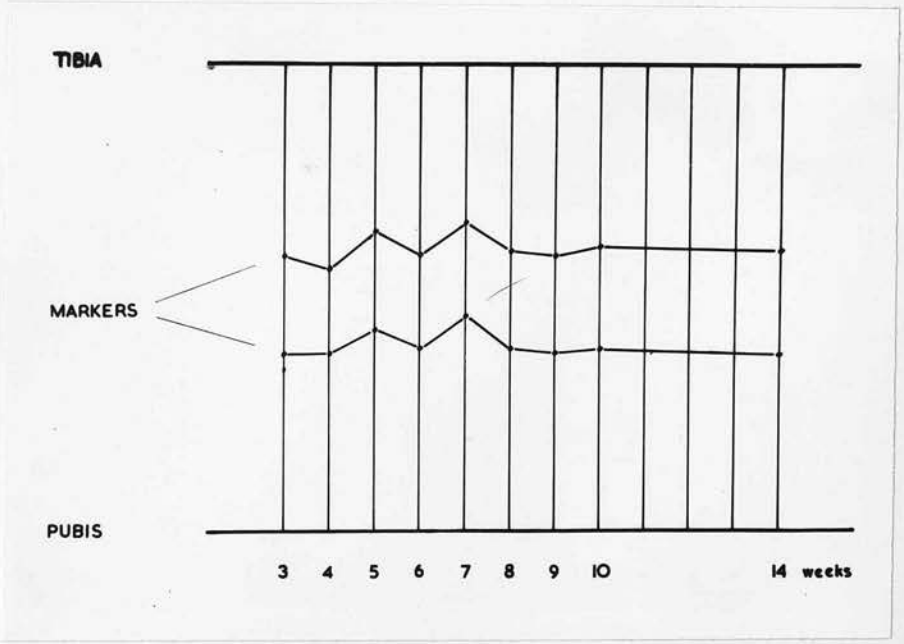
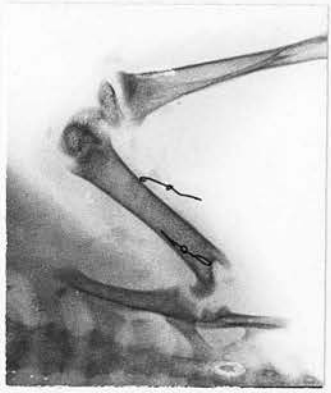


Positions of Markers



Anterior Gracilis 3



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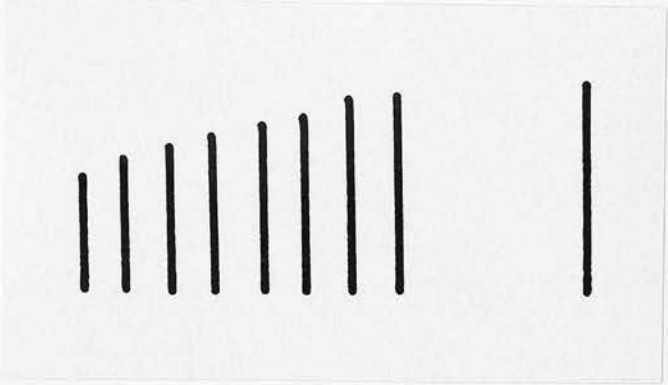
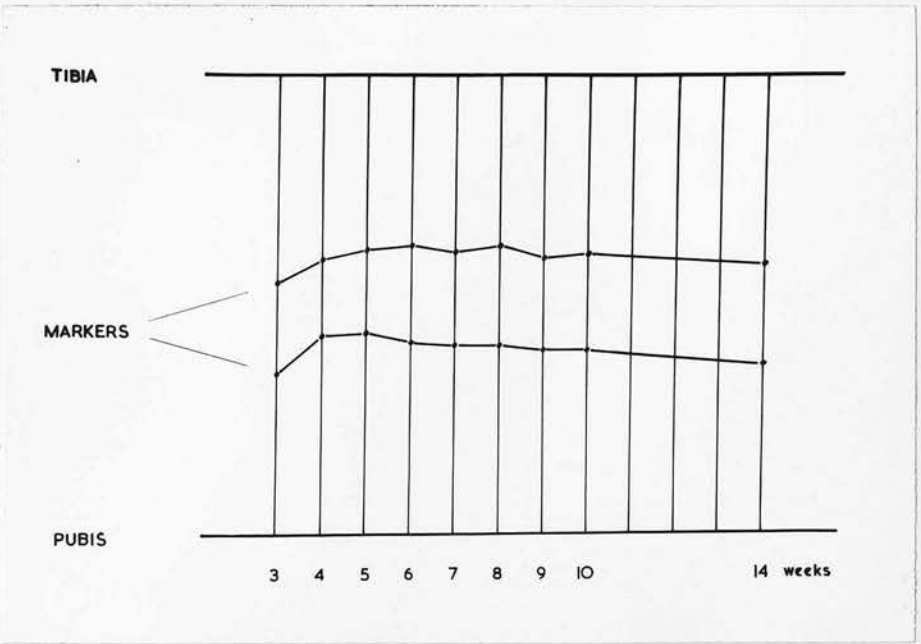
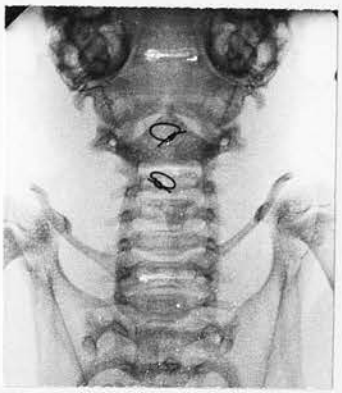


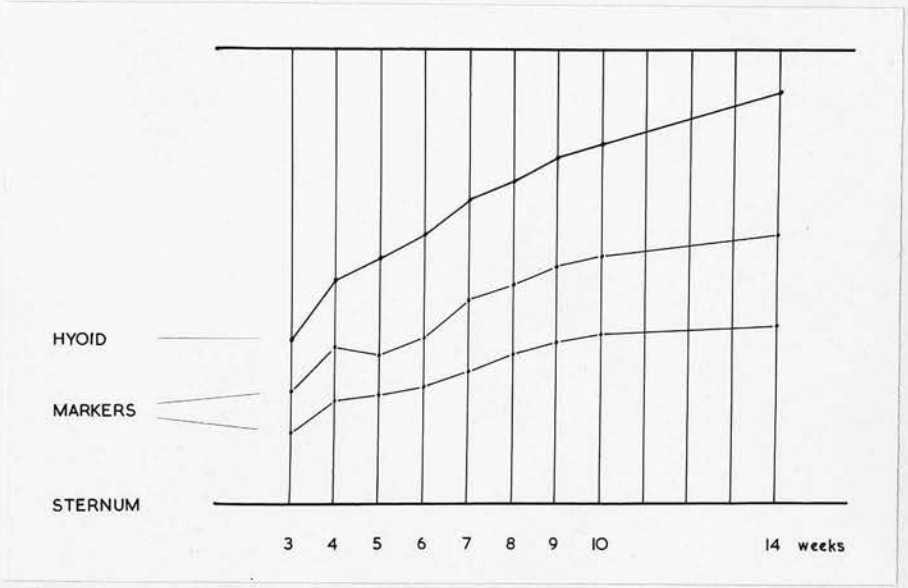
Positions
of
Markers



Sterno-Hyoid 1



Absolute positions of markers and ends of muscle. (x 2 approx.)



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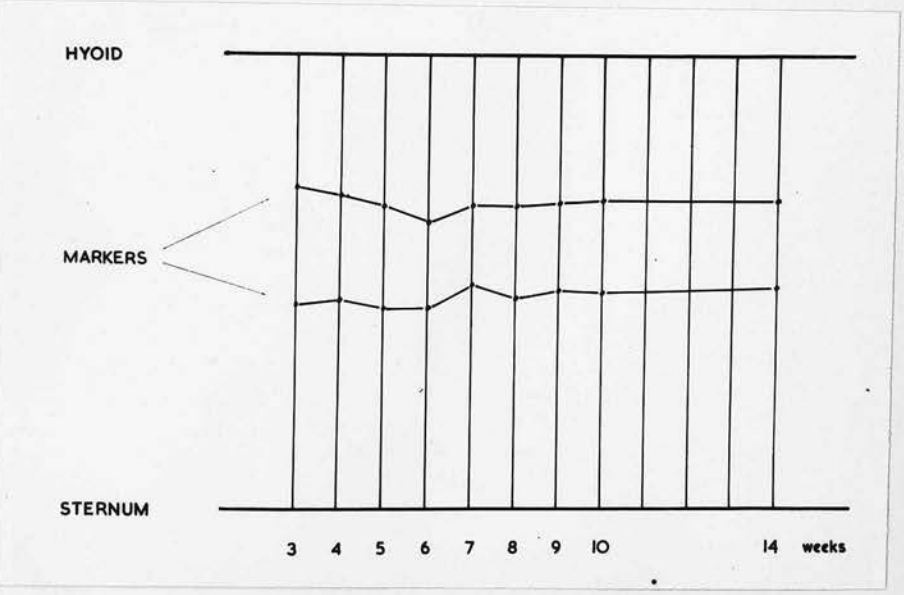
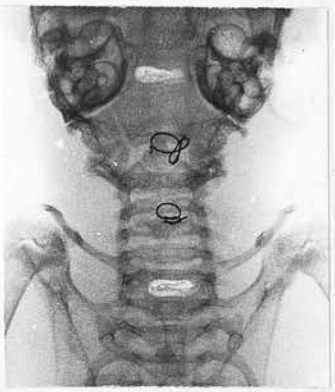
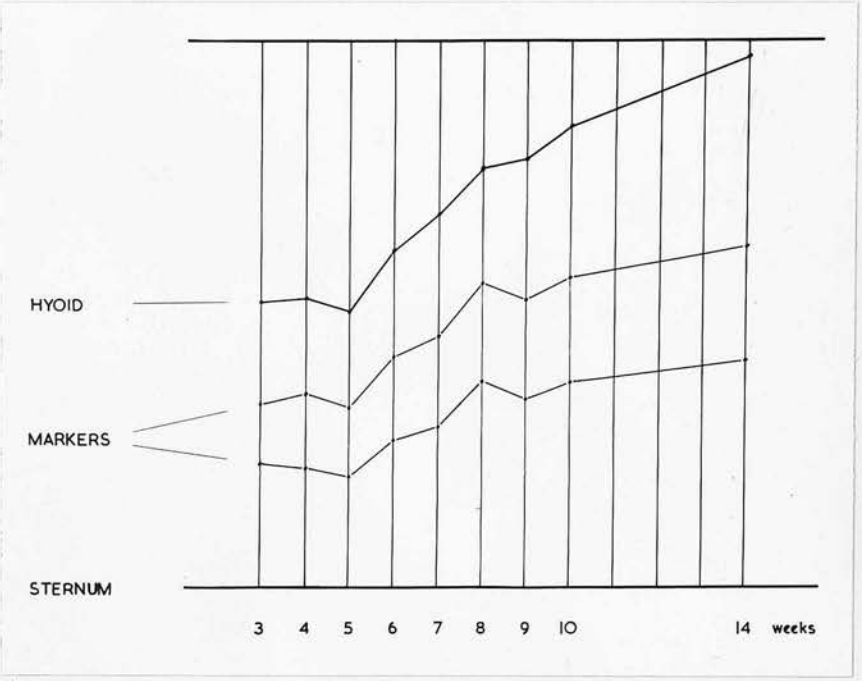


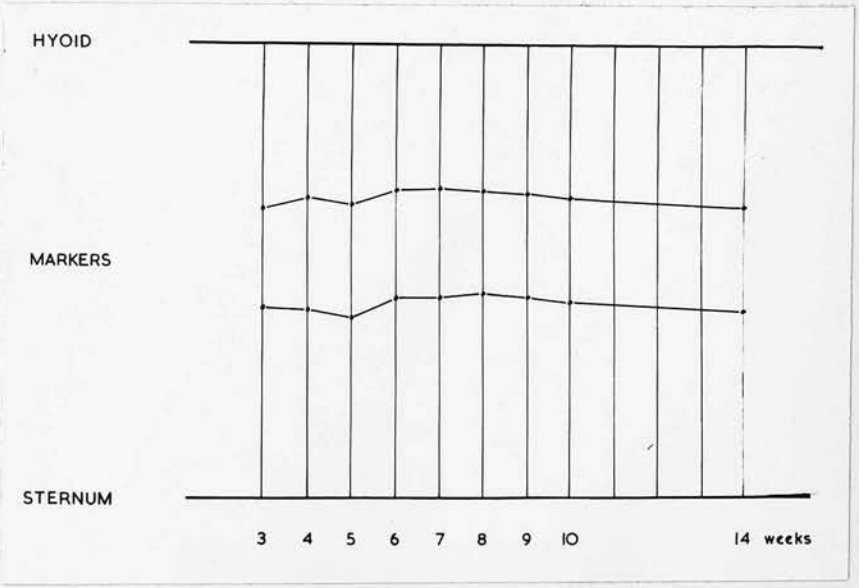
Sterno-Hyoid 2



Absolute positions of markers and ends of muscle. (x 2 approx.)

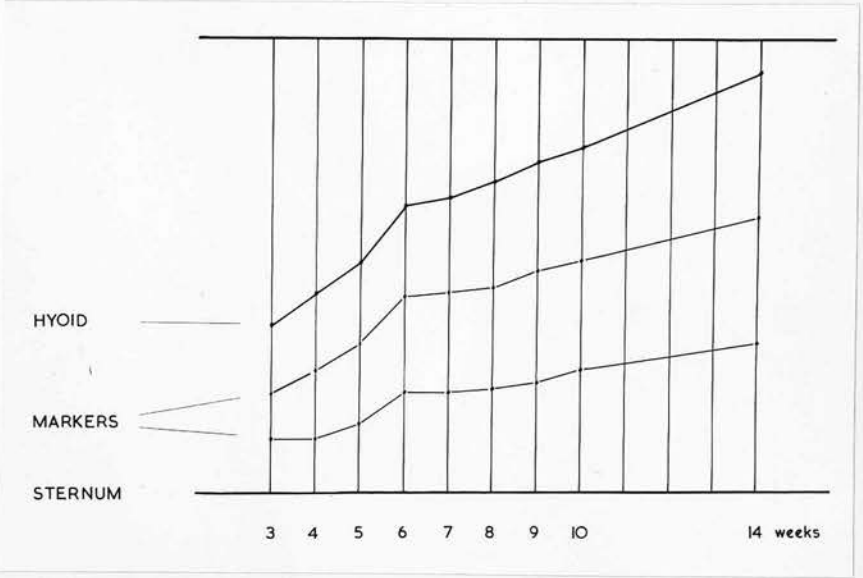


Relative positions of markers and ends of muscle.

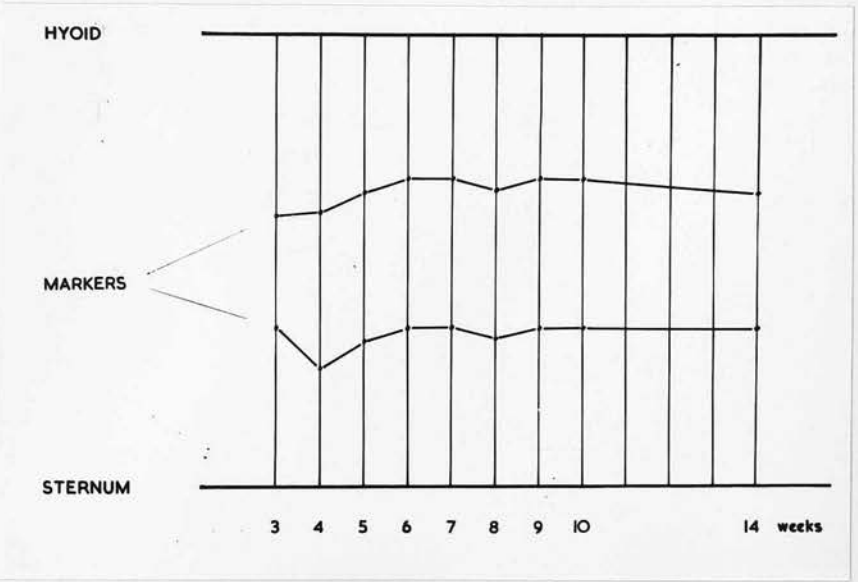


Sterno-Hyoid 3

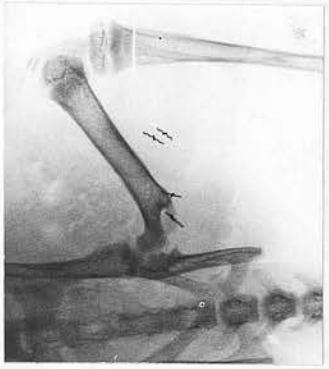
Absolute positions of markers and ends of muscle. (x 2 approx.)



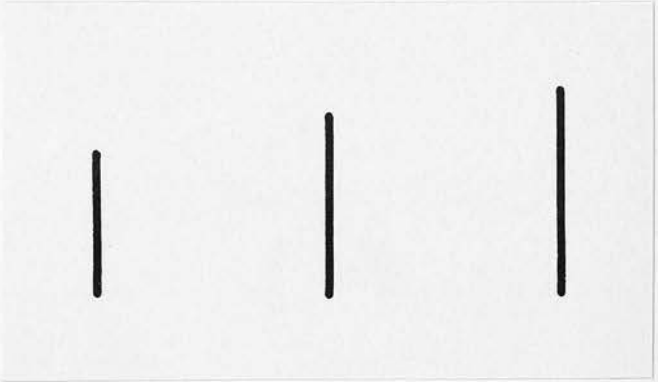
Relative positions of markers and ends of muscle.



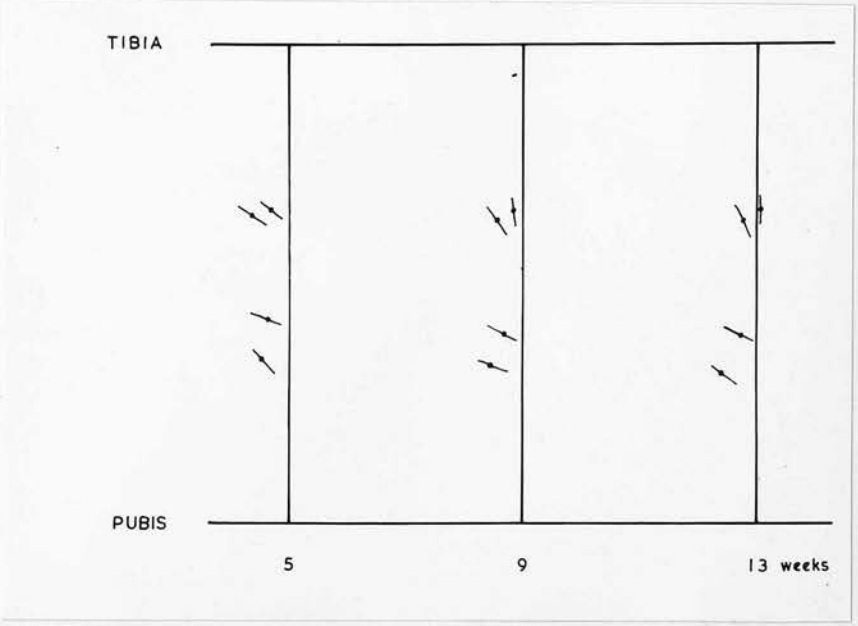
Anterior Gracilis 4



Femur length

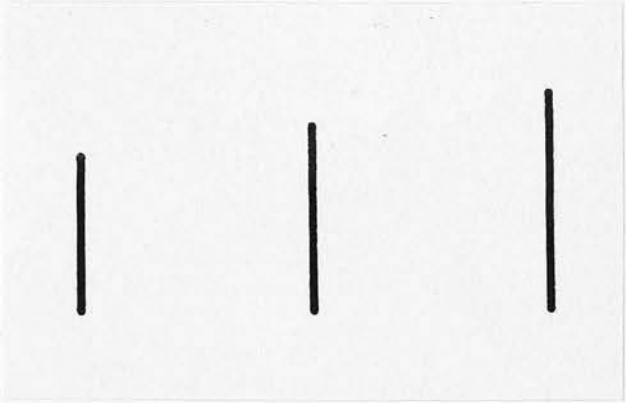


Positions of Markers

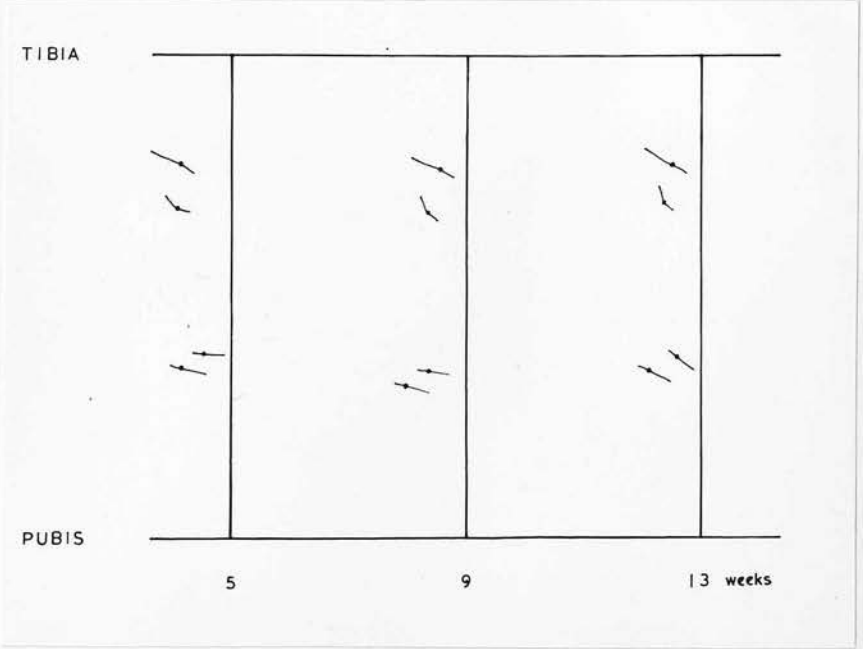


Anterior Gracilis 5

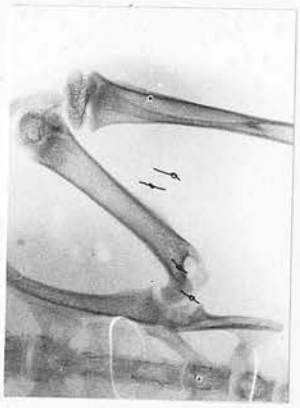
Femur length



Positions
of
Markers



Anterior Gracilis 7



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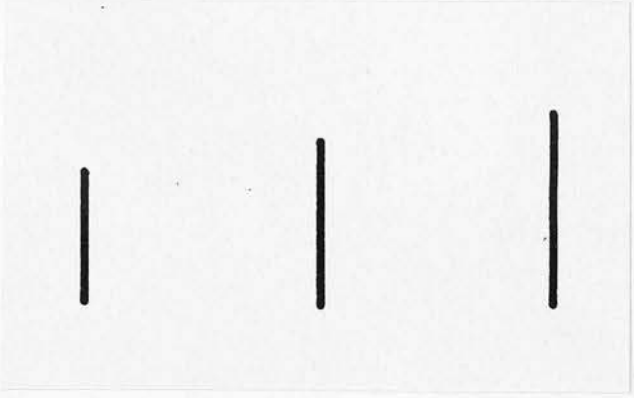
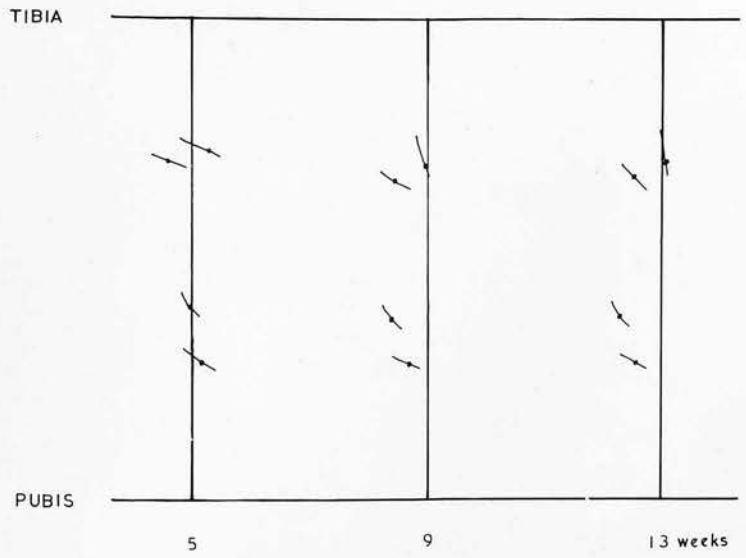
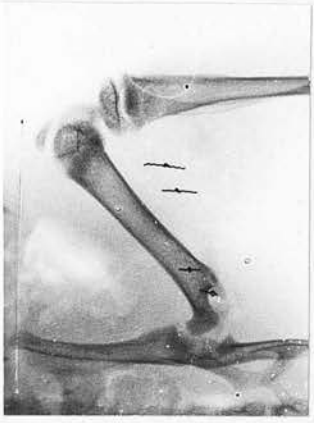


Positions of Markers



Anterior Gracilis 8



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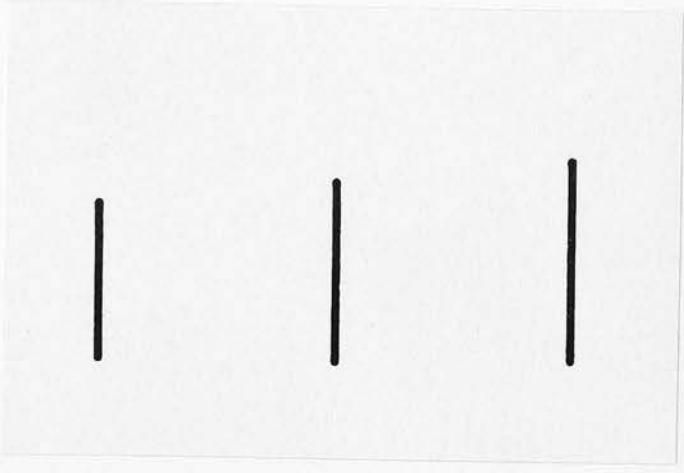

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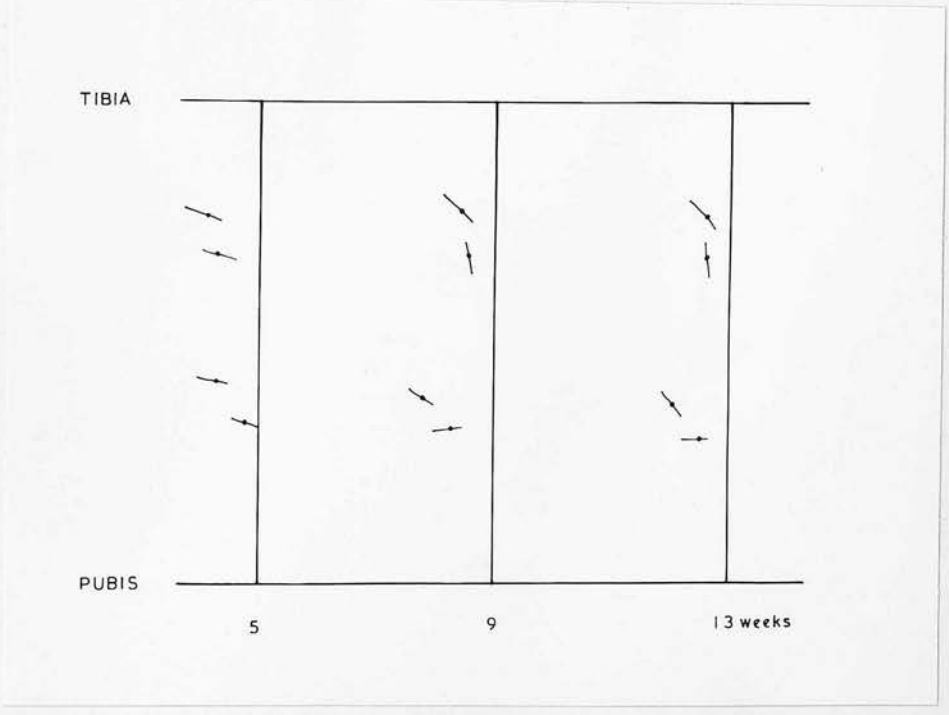

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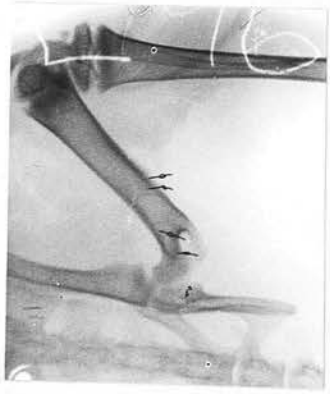

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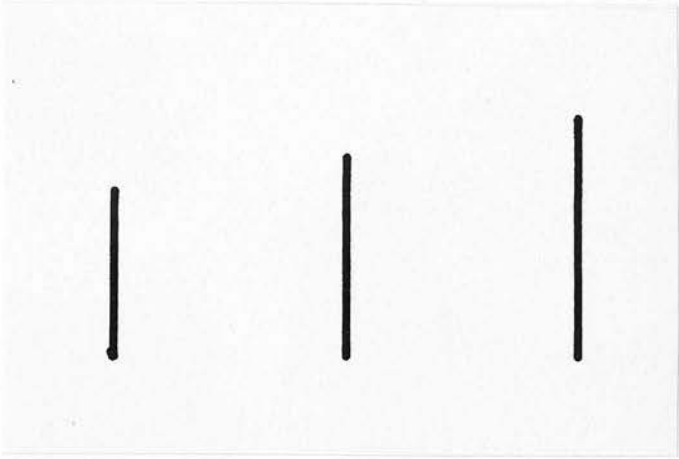

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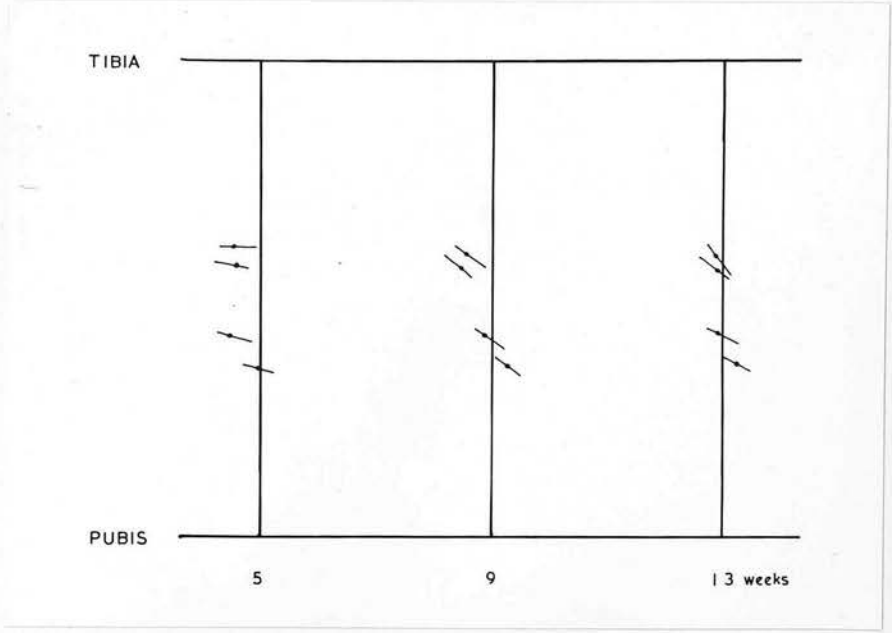
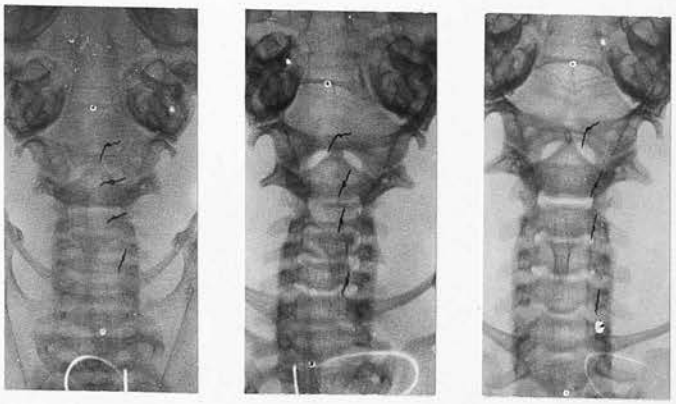


Sterno-Hyoid 4

X-rays of Markers



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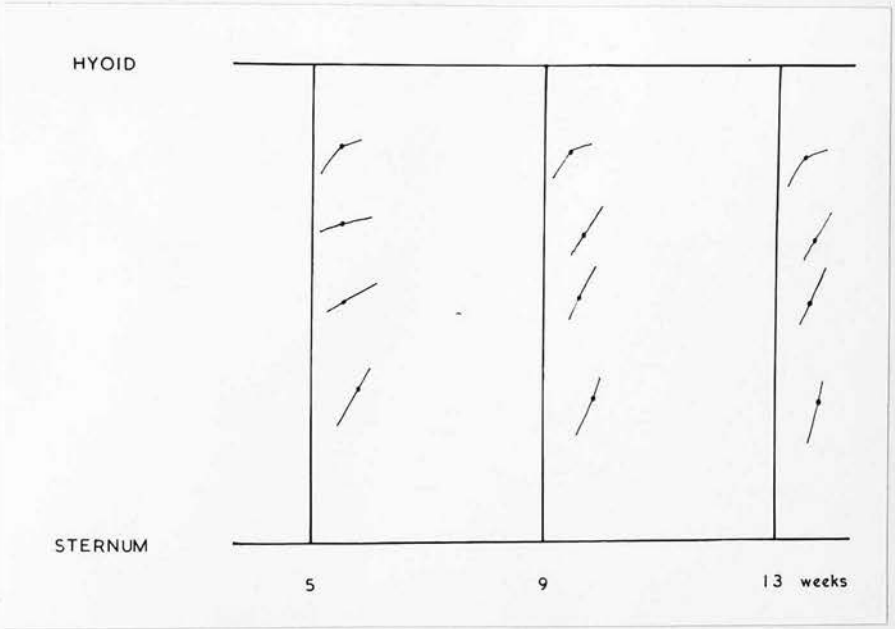
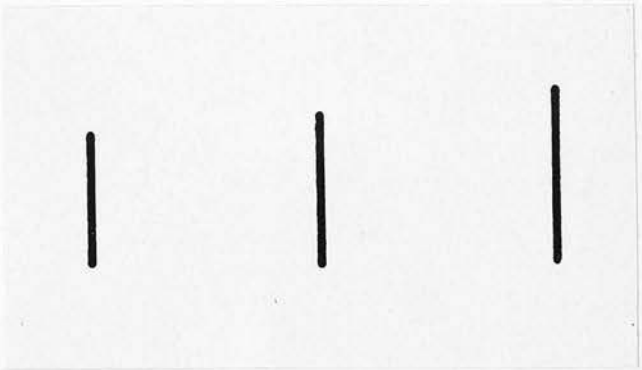


Sterno-Hyoid 5

Muscle length



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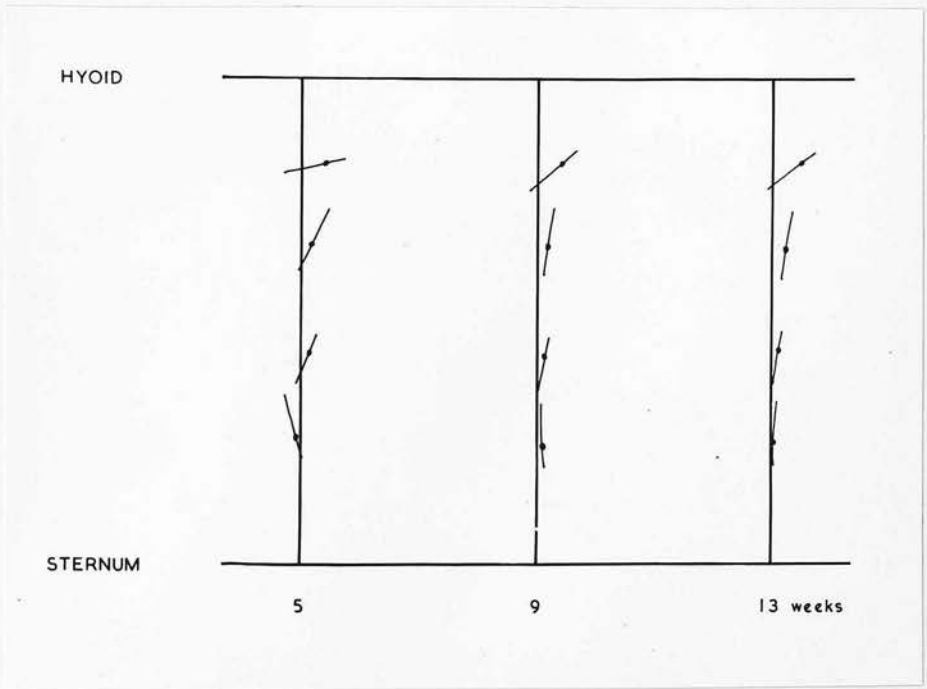

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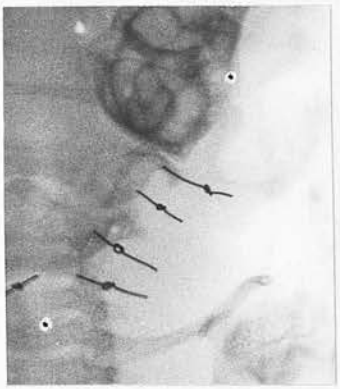
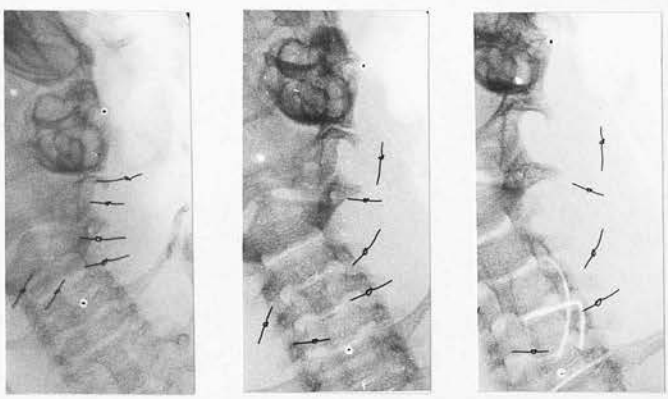
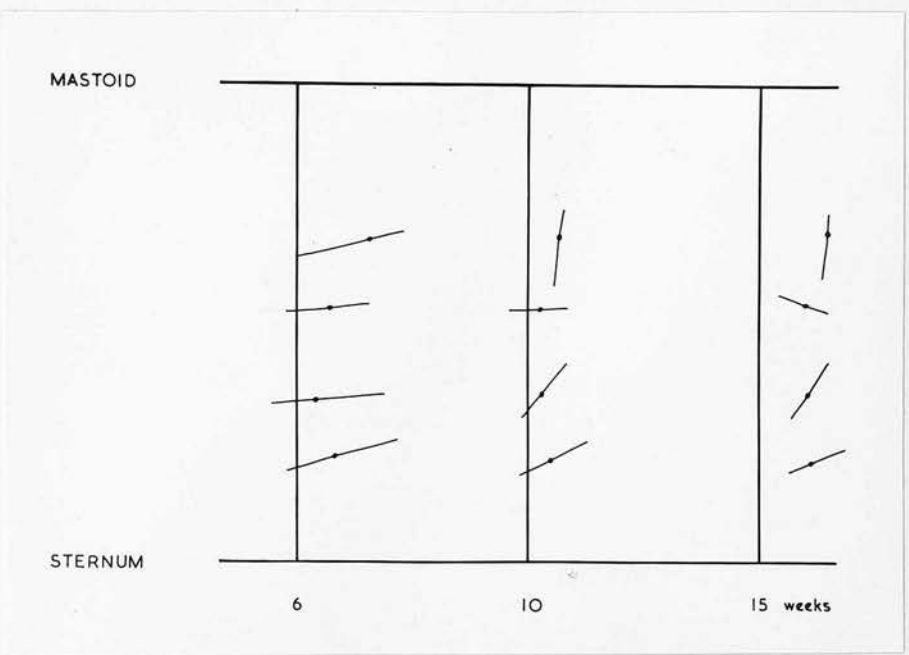


X-rays of Markers



Positions of Markers



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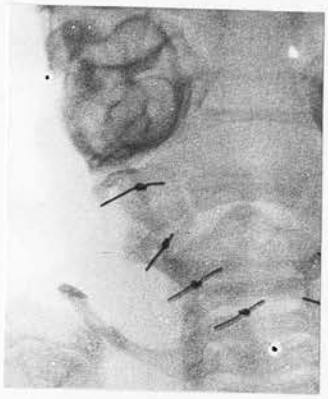
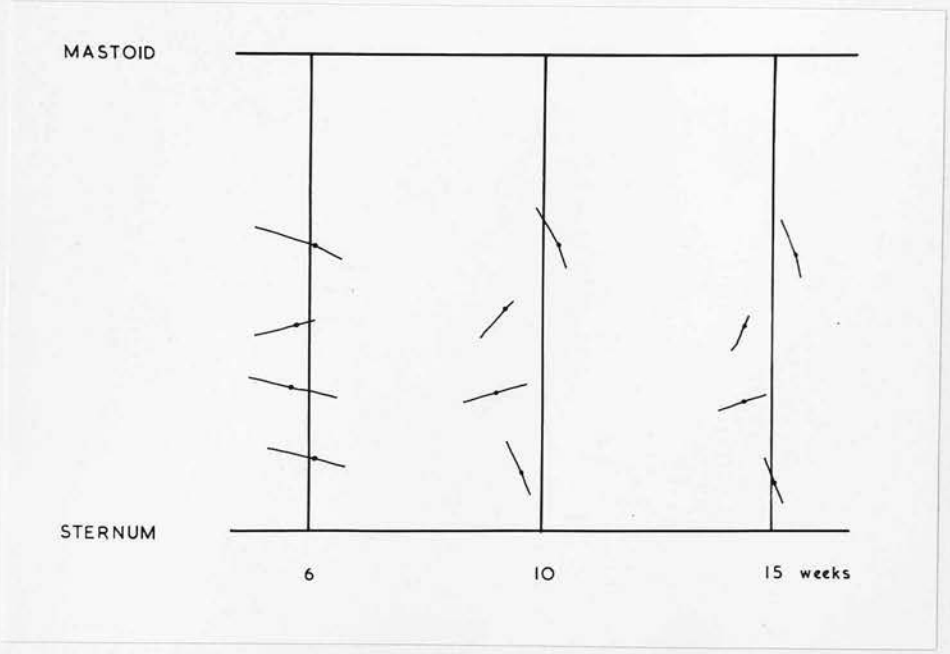


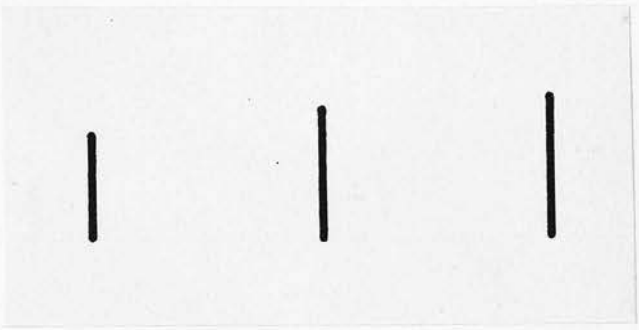
Muscle length

Positions of Markers

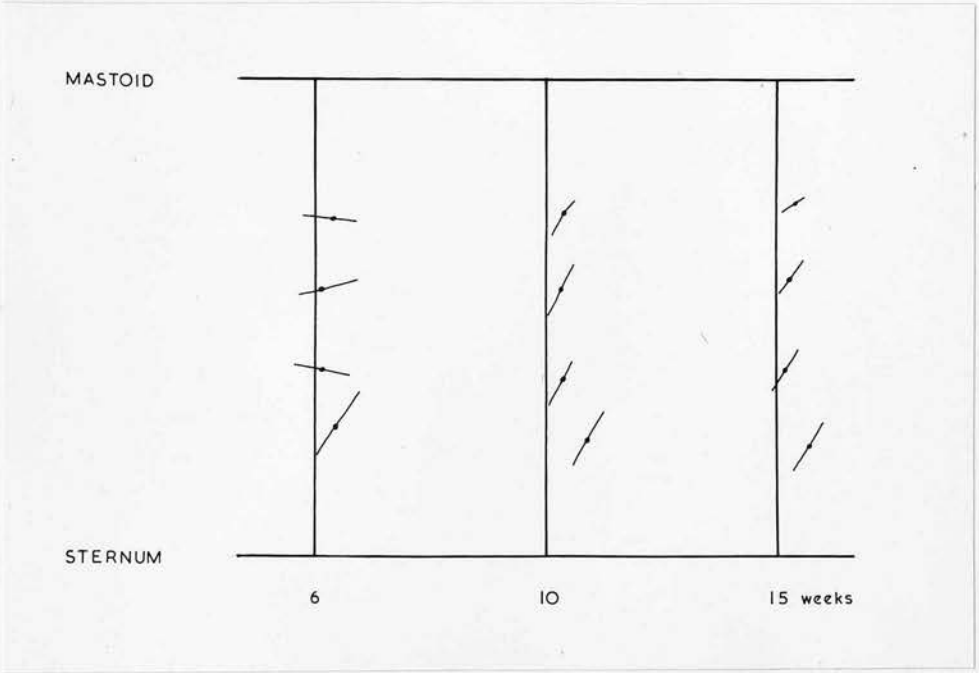


Sterno-Mastoid 3

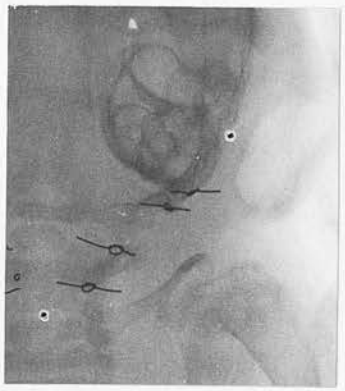
Muscle length



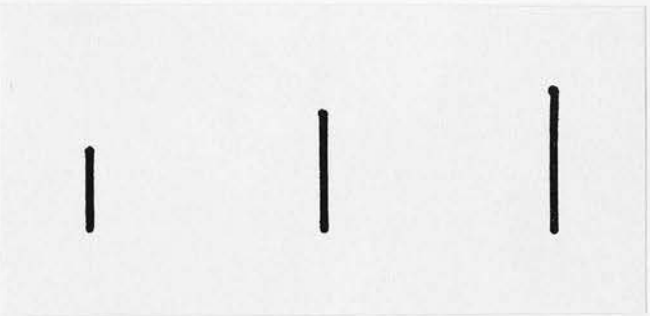
Positions of Markers



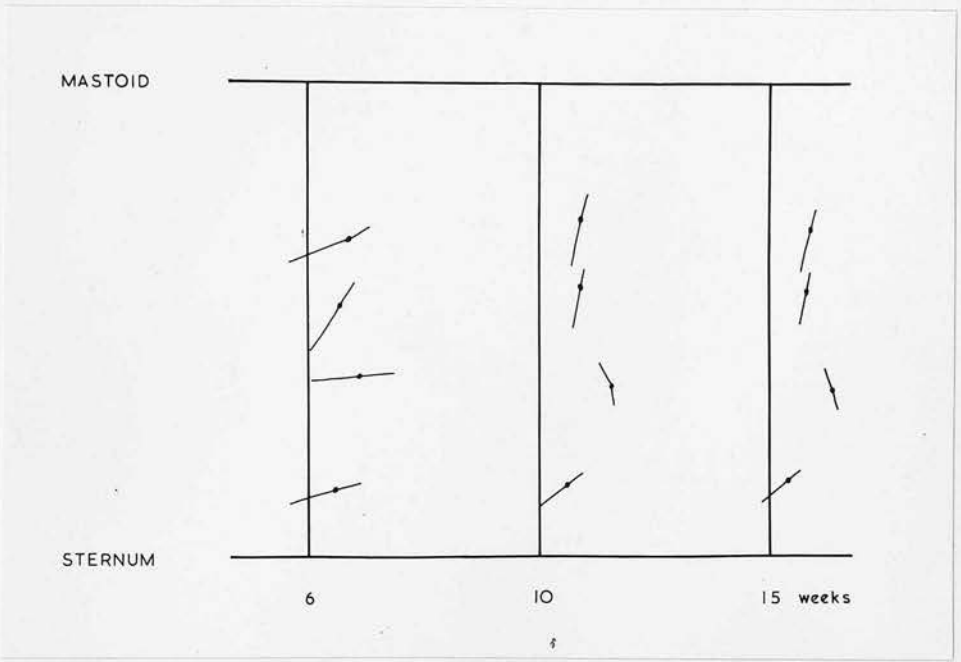
Sterno-Mastoid 4



Muscle length



Positions of Markers



Acknowledgements

I wish to thank Professor G. J. Romanes for giving me the opportunity to carry out this work, and for his constant interest and advice in the course of it.

I am particularly grateful to Dr. A. R. Muir for his patient supervision, guidance, and encouragement; and to Mr. D. Adams, Mr. J. Campbell, Dr. B. L. Ginsborg, and Dr. A. Peters, for their invaluable help. I am indebted to Dr. J. B. King for his expert radiographic advice, to many others who have given me the benefit of their opinions and expert knowledge, and for many gifts of material. Mr. H. Tully has rendered valuable photographic assistance, and the electron microscope, which is on loan from the Wellcome Trust, is maintained by Mr. George Wilson.

Finally, may I thank Dr. Marie Barker and Fräulein Dagmar Leonhard for their willing help with German translations.

Communications based on results recorded in this work have been delivered to the Anatomical Society (1959), to the Physiological Society (1960), to the Première Réunion Européenne d'Anatomie (Strasbourg, 1960), and to a Symposium on Cholinesterase held at the University of Basle (1960).

A Paper, "Observations on the terminal innervation of segmental muscle fibres in amphibia", was published with A.R. Muir and A. Peters in Acta Anatomica, Vol. 40 : p. 1.

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